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(71) Applicant: IMARx PHARMACEUTICAL CORP. [US/US]; 1635 E. 18th Street, Tucson, AZ 85719 (US).

(72) Inventors: UNGER, Evan, C.; 13365 E. Camino La Cebadilla, Tucson, AZ 85749 (US). MCCREERY, Thomas; 1430 N. Rosemont Boulevard, Tucson, AZ 85712 (US). SADE-WASSER, David; 3493B Reynard Way, San Diego, CA 92103 (US).

(74) Agents: MILLER, Suzanne, E. et al.; Woodcock Washburn Kurtz Mackiewicz & Norris LLP, 46th floor, One Liberty Place, Philadelphia, PA 19103 (US).

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(54) Title: A METHOD OF INCREASING NUCLEIC ACID SYNTHESIS WITH ULTRASOUND

(57) Abstract

The present invention is directed to a method of increasing nucleic acid synthesis in a cell comprising administering to the cell a therapeutically effective amount of ultrasound for a therapeutically effective time such that said administration of said ultrasound results in said increased nucleic acid synthesis. The nucleic acid sequence may comprise an endogenous sequence or an exogenous sequence.

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A METHOD OF INCREASING NUCLEIC ACID SYNTHESIS WITH ULTRASOUND

Related Applications

This application is a continuation-in-part of U.S. application Serial No. 08/841,169, filed April 29, 1997, which is a continuation-in-part of U.S. application Serial No. 08/785,661, filed January 17, 1997, which in turn is a continuation-in-part of U.S. application Serial No. 08/640,554, filed May 1, 1996, now abandoned, the disclosures of each of which are hereby incorporated herein by reference in their entireties.

Field of the Invention

This invention relates to the field of gene expression, in particular, to the use of ultrasound to increase synthesis of a nucleic acid sequence in a cell.

Background of the Invention

The regulation of gene expression in cells has been a topic of considerable research interest since the earliest discoveries on the central dogma of the role of DNA and RNA in protein synthesis. The earliest models were developed in procaryotic systems and involved genetic elements such as promoters, operators and repressors. At the same time, in higher organisms, early observations demonstrated that certain genes appeared to be permanently repressed and unexpressed in certain tissues, others were more or less perpetually active, and still others were subject to elaborate and complex regulatory control, influenced

by environmental, hormonal, and age-related factors. In view of the postulation of secondary messenger theories in the mid-1980s, some degree of molecular basis began to be understood for environmental influences on regulating gene expression. Still, to date, a detailed understanding of regulation of gene expression resulting from many environmental factors is largely speculative.

One of the areas of interest in understanding how genetic processes may be influenced by external factors involves what happens when cells are exposed to external energy fields such as different intensities and frequencies of electromagnetic energy. While there appears to be little consensus on the effect of external electromagnetism on living organisms, exposure to ultrasound has received little study on the cellular level. Few published reports are available. Tuncay *et al.*, *J. Dental Res.* **1997** *76*:2903 and Tuncay *et al.*, *J. Dental Res.* **1996** *75*:143, abstract no. 1007 recently reported the effects of ultrasound on endogenous proto-oncogene and structural gene expression in gingival fibroblasts. Yang *et al.*, *J. Orthopaedic Res.* **1996** *14*(5):802-9 show an effect on bone tissue repair when bone fractures were exposed to ultrasound. The genetic expression this group found to be stimulated were primarily structural genes such as those for procollagen. Unlike the reports of Tuncay *et al.* and Yang *et al.*, the present invention exhibits a statistically significant increase in synthesis of nucleic acid sequences upon exposure to ultrasound. Thus, it appears, that genes which are normally functional only in times when repair is the predominant mode are affected by ultrasound. These findings have profound therapeutic consequences to induce cell repair in tissue under disease conditions.

Summary of the Invention

The present invention is directed, *inter alia*, to a method for increasing nucleic acid synthesis in a cell comprising administering to the cell a therapeutically effective amount of ultrasound for a time sufficient to result in increased synthesis of an endogenous nucleic acid sequence encoding a stress protein and repair protein.

A method of increasing nucleic acid synthesis in a cell comprising administering to the cell a nucleic acid sequence and a therapeutically effective amount of ultrasound for a time sufficient to result in increased synthesis of an exogenous nucleic acid sequence is also contemplated by the present invention.

Also embodied by the present invention is a method of identifying a nucleic acid sequence exhibiting increased synthesis comprising administering to a cell ultrasound for a time sufficient to increase synthesis of a nucleic acid sequence, and observing an increase in synthesis of said nucleic acid sequence.

Further, the invention pertains to a method of treating a condition in a human subject comprising administering ultrasound to the subject for an amount of time sufficient to increase synthesis of a nucleic acid sequence, wherein the increased synthesis of a nucleic acid sequence results in treating said subject.

These, as well as other, aspects of the invention are set forth in greater detail below.

Brief Description of the Figures

FIGURE 1 is a graph showing relative levels of mRNA as a function of time in NIH-3T3 cells exposed to ultrasound.

FIGURE 2 is a photograph of electrophoresis polymerase chain reaction (PCR)-amplification samples showing amplified cDNA in Figure 2A without ultrasound and Figure 2B with ultrasound for 33 PCR primers.

Detailed Description of the Invention

As employed above and throughout the disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings.

"Ultrasound", "Sonoporation™", and similar terms, refer to pulses of sound energy, preferably repetitive pulses, sufficient to result in increasing synthesis of a nucleic acid sequence in a cell. Preferably, the ultrasound is in the frequency range of from about 10 kilohertz to less than about 50 megahertz and at an energy level of from about 200 milliwatts/cm^2 to about 10 watts/cm^2. While not intending to be bound to any particular theory of operation, the ultrasound may also assist in the uptake of an exogenous nucleic acid sequence by a cell by inducing openings in the cell membrane, or perhaps bursting endosomes inside a cell allowing compounds to escape. Indeed, cells may be induced to take up (e.g., be transfected with) nucleic acid sequences with ease compared to conventional methods. Typically the ultrasound is applied by external application, via a standard clinical ultrasound

device, but may also be applied in other fashions, such as endoscopically, intravascularly, and with a portable ultrasound device, such as one worn by the subject. If desired, in certain applications, ultrasound may also result in the formation of a gas from a gaseous precursor.

"Nucleic acid" and "nucleic acid sequence" include nucleotides, nucleosides, DNA including genomic DNA and cDNA, RNA, antisense sequences, oligonucleotides, and the like. Single and double stranded nucleic acid sequences, including and not limited to oligonucleotide sequences of about 100 kb to about 1,000,000 kb (including whole chromosomes), preferably of about 4 kb to about 6 kb, more preferably about 1,000 nucleotides in length, more preferably about 500 nucleotides in length, more preferably about 250 nucleotides in length, more preferably about 100 nucleotides in length, more preferably about 50 nucleotides in length, more preferably about 25 nucleotides in length, more preferably about 10 nucleotides in length, even more preferably about 3 kbp to about 10 kbp in length, are contemplated by the present invention. Embodied by the term "nucleic acid sequence" are all or part of a gene, at least a portion of a gene, a gene fragment, a sense sequence, an antisense sequence, an antigene nucleic acid, a phosphorothioate oligodeoxynucleotide, and an alteration, deletion, mismatch, transition, transversion, mutation, conservative substitution, and homolog of a sequence. The phrase "at least a portion of," and "all or part of," as used herein, means that the entire gene need not be represented by the sequence so long as the portion of the gene represented is effective to block or exhibit, depending on the type of sequence used, gene expression. The sequences may be incorporated into an expression vector such as, and not limited to, a plasmid, phagemid, cosmid, yeast artificial chromosome (YAC), virus (e.g., adenovirus, vaccinia virus, retrovirus), and defective virus (also known as a "helper virus"). The nucleic acid sequence may also be administered naked, that is without an expression vector.

Variations in the nucleic acid and polypeptide sequences of the present invention are within the scope of the present invention and include N terminal and C terminal extensions, transcription and translation modifications, and modifications in the cDNA sequence to facilitate and improve transcription and translation efficiency. In addition, mismatches within the sequences identified herein, which achieve the methods of the invention, such that the mismatched sequences are substantially complementary to the

sequences identified, are also considered within the scope of the present invention. Mismatches which permit substantial complementarity to particular sequences, such as similarity in residues in hydrophobicity and hydrophilicity, will be known to those of skill in the art once armed with the present disclosure. In addition, the sequences of the present invention may be natural or synthetic. Homologs and alternatively spliced sequences and fragments of the sequences which are substantially similar or have substantially the same activity of the sequences of the present invention are also contemplated herein.

ENDOGENOUS AND EXOGENOUS SEQUENCES

Endogenous and exogenous sequences are contemplated by the present invention. Endogenous sequences are sequences that naturally arise from a cell. Exogenous or heterologous sequences denote a nucleic acid sequence that is not obtained from and would not normally form a part of the genetic make-up of a cell. Accordingly, an exogenous sequence may be a sequence from another source added to a particular cell.

For purposes of the present invention, endogenous nucleic acid sequences which exhibit increased synthesis upon ultrasound include nucleic acid sequences encoding stress proteins and repair proteins. Stress proteins refer to proteins induced upon environmental insult to the cell, such as heat, cold, metals including heavy metals, and the like. Stress proteins include, for example, heat shock proteins, heat shock protein 60, heat shock protein 27, and heat shock protein 89α, for example. Repair proteins denote proteins involved in cellular repair mechanisms, such as for cellular upkeep. Repair proteins include proteins, typically enzymes, such as nucleases for DNA repair, for example endonucleases and exonucleases, DNA and RNA polymerases, Rnases including E, F, H, P, T, and III.

In the case of exogenous nucleic acid sequences and in accordance with the methods of the present invention, the synthesis of any nucleic acid sequence added to a cell is increased by ultrasound. Accordingly, nucleic acid sequences encoding stress proteins, repair proteins, as well as structural proteins, proto-oncogenes, and regulatory proteins, for example, exhibit increased synthesis upon administration of the same and ultrasound to a cell. Structural nucleic acid and protein sequences encode products, such as protein, enzymes, tRNA, as opposed to a nucleic acid or protein sequence that serves a regulatory role. Proto-oncogenes are the normal, cellular equivalent of an oncogene, involved in the signaling or

regulation of cell growth. An oncogene is a mutated and/or overexpressed proto-oncogene of animal cells that in a dominant fashion releases the cell from normal restraints on growth, and alone, or in concert with other changes, converts a cell into a tumor cell. Proto-oncogenes useful in the present invention include and are not limited to *c-fos, c-myc, c-jun*, and *jun-b*. Regulatory nucleic acid sequences encode molecules controlling the sequencing or expression of a gene, and include promoters, enhancers, and the like.

Nucleic acid sequences encoding proteins, proto-oncogenes, oncogenes, and the like include and are not limited to, initiation factor 3 (IF3), tRNA synthetases, heat shock proteins (hsp) including hsp 27 (SEQ ID NOS: 29 and 30), hsp 60 (SEQ ID NOS: 31 and 32), hsp 89α (SEQ ID NOS: 37 and 38), ubiquinone oxidoreductase (SEQ ID NOS: 57 and 58), xeroderma pigmentosum A (XPA) nucleotide excision repair gene (SEQ ID NOS: 61 and 62), xeroderma pigmentosum B (XPB) nucleotide excision repair gene (SEQ ID NOS: 63 and 64), XPG nucleotide excision repair gene (SEQ ID NOS: 65 and 66), Ca+2 ATPase (SEQ ID NOS: 19 and 20), ERCCl (SEQ ID NOS: 23 and 24) a locus on human chromosome 19, Heme Oxygenase (SEQ ID NOS: 35 and 36), Rad23 (SEQ ID NOS: 45 and 46) also known as HHR23B, a yeast DNA repair gene, Raf (SEQ ID NOS: 47 and 48) an oncogene, TCP-1-B (SEQ ID NOS: 55 and 56) T complex protein 1-B, calsequestrin (SEQ ID NOS: 7 and 8), β-polymerase (SEQ ID NOS: 13 and 14), 3-methyladenine DNA glycosylase (SEQ ID NOS: 15 and 16), MAP-kinase kinase (SEQ ID NOS: 41 and 42) microtubule associated protein kinase kinase, pericentrin (SEQ ID NOS: 43 and 44), ubiquitin (SEQ ID NOS: 59 and 60), B2 (SEQ ID NOS: 3 and 4), Cox3 (SEQ ID NOS: 17 and 18), erg-1 (SEQ ID NOS: 25 and 26), HHR6B (SEQ ID NOS: 27 and 28), HHR6A (SEQ ID NOS: 33 and 34), RPA (SEQ ID NOS: 49 and 50), SRC (SEQ ID NOS: 53 and 54), catalase (SEQ ID NOS: 11 and 12), creatine kinase (SEQ ID NOS: 21 and 22), oncogenes: Ras (SEQ ID NOS: 51 and 52), c-fos (SEQ ID NOS: 1 and 2), c-myc (SEQ ID NOS: 5 and 6), c-jun (SEQ ID NOS: 9 and 10), and jun-b (SEQ ID NOS: 39 and 40).

tRNA synthetases include and are not limited to alanyl, arginyl, asparagyl, aspartyl, cystyl, glutamyl, glycyl, histidyl, isoleucyl, leucyl, lysyl, methionyl, phenylalanyl, prolyl, seryl, threonyl, tryptophyl, tyrosyl, and valyl tRNA synthetase.

Methods of introducing exogenous sequences into a cell (also referred to variously herein as methods for delivering a sequence into a cell, methods of intracellular

delivery, methods of promoting, effecting, facilitating or enhancing the uptake of a sequence into a cell, and the like) include "transfection", which refers to the introduction of genetic material, i.e., a nucleotide sequence (e.g., DNA or RNA) into a host cell. Transfection is also sometimes referred to as transformation. DNA (or RNA) which is new to the cell into which it is incorporated is typically referred to as heterologous DNA (or RNA) or exogenous DNA (or RNA). Some bacterial species take up exogenous DNA and do not discriminate between uptake of DNA from a similar or same species or from a completely different species or organism. Exogenous DNA may also be taken up by cells, but may or may not be incorporated into nuclear material in a hereditable manner. The objective of transfection of a host cell may be to effect expression of one or more carefully selected sequences.

Increased synthesis refers to transcription and/or translation of a nucleic acid sequence resulting in the production of an amino acid, peptide and/or protein. Upon administration of ultrasound, in the case of endogenous sequences, synthesis of the nucleic acid sequence may be increased (promoted, enhanced), and in fact may be enhanced in that the expression of the nucleic acid sequences as compared to the natural synthesis of the particular nucleic acid sequence is increased. In the case of exogenous sequences, administration of the nucleic acid sequence and ultrasound results in increased synthesis of the sequence as compared to conventional transfection techniques such as calcium phosphate precipitation, viral vectors, microinjection, shock wave such as for example lithotripsy, and electroporation, may be increased. Methods of measuring increased nucleic acid synthesis will be known to skilled artisans once armed with the present disclosure and include amplification and enzyme-linked immunosorbent assay (ELISA) as well as methods disclosed in Sambrook, *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989), the disclosures of which are hereby incorporated herein by reference in their entirety. Thus, as a result of the methods of the present invention, a product (e.g., a nucleic acid sequence or a protein) may be produced. In addition, the prevention of the production of a product (such as, as a result of an antisense sequence delivered into the cell) by the host cell may also result.

Without being bound by any theory of operation, it is believed that ultrasound may also induce a cell to take up an exogenous nucleic acid sequence in accordance with the methods of the present invention. Included within the definition of delivery of a sequence

into a cell in accordance with the methods of the present invention are active and passive mechanisms of cellular uptake. Ion channels and other means of transport utilized by cells to incorporate extracellular materials, including compounds to be delivered thereto, into the intracellular milieu are encompassed by the present invention.

"Cell" and "host cell" refer to prokaryotic cells and eukaryotic cells, including plant cells, animal cells, cells of unicellular organisms, cells of multicellular organisms, etc. Especially preferred are animal cells, more preferably mammalian cells and most especially human cells, including but not limited to living cells, tissues, and organs. Eukaryotic cells are cells of higher organisms in which genetic material is enclosed by a nuclear membrane. Prokaryotic cells are cells of lower organisms that lack a well defined nucleus and contain genetic material that is not enclosed within a membrane of its own. The cells may be present *in vivo* or *in vitro* (e.g. in cell culture).

The invention has wide applications for increasing (promoting, facilitating or enhancing) nucleic acid synthesis in both *in vitro* and *in vivo* applications, and is particularly useful for prokaryotic and eukaryotic animal cells, particularly mammalian cells. Intracellular delivery includes delivery into the cells through a cell membrane (plasma membrane), cell wall, and/or nuclear membrane. In the case of exogenous nucleic acid sequences, the efficiency of intracellular delivery (e.g., transfection) of such nucleic acid sequences is also increased.

The phrase "cell membrane" (also termed "plasma membrane") is used in its conventional sense as denoting the outer layer or boundary of the cytoplasm of a living cell. Cell membranes are typically comprised of protein and lipids, and are generally found in animal cells.

The phrase "cell wall" is also used in its conventional sense to denote a rigid or semi-rigid outer covering surrounding the protoplasts of plant cells and most prokaryotes. Cell walls are typically found, for example, in cells of bacteria, plants, algae, and fungi. Cell walls are, on the other hand, generally not present in animal cells. In plants, the wall typically comprises several layers; a primary wall composed of cellulose microfibrils running through a matrix of hemicelluloses and pectic substances surrounded by a secondary wall composed of cellulose which is generally lignified to a varying extent. Cell walls of fungi may contain

varying amounts of chitin. Cell walls of prokaryotes are typically strengthened by mucopeptides and may be surrounded by a mucilagenous capsule.

A wide variety of nucleic acid sequences may be delivered as bioactive agents, diagnostic agents, pharmaceutical agents, and the like. Whole genes, multiple gene sequences, and gene fragments may be utilized as well as whole chromosomes and chromosome fragments.

METHODS OF OBSERVING INCREASED SYNTHESIS OF A TARGET NUCLEIC ACID SEQUENCE

Amplification incudes the use of at least one primer sequence which is complementary to a portion of nucleic acid sequence the synthesis of which is to be increased. Where a template dependent process of amplification uses a pair of primers, one primer of the pair may comprise oligonucleotides which are complementary to a nucleic acid sequence the synthesis of which is increased upon ultrasound application.

Alternatively, each of the two oligonucleotides in the primer pair may be specific to a nucleic acid sequence the synthesis of which is increased upon ultrasound application. The primers may be designed to be complementary to separate regions of the subject sequence, for example. By separate regions is meant that a first primer is complementary to a 3' region of the subject sequence and a second primer is complementary to a 5' region of the subject sequence. Preferably, the primers are complementary to distinct, separate regions and are not complementary to each other. Any primer pairs which transcribe nucleic acids toward each other and which are specific for a subject sequence may be used in accordance with the methods of the present invention.

Total extraction of RNA is preferably carried out. As used herein, the term "amplification" refers to template-dependent processes and vector-mediated propagation which result in an increase in the concentration of a specific nucleic acid molecule relative to its initial concentration, or in an increase in the concentration of a detectable signal. As used herein, the term template-dependent process is intended to refer to a process that involves the template-dependent extension of a primer molecule. The term template dependent process refers to nucleic acid synthesis of an RNA or a DNA molecule wherein the sequence of the newly synthesized strand of nucleic acid is dictated by the well-known rules of complementary base pairing (see, for example, Watson, J. D. et al., In: Molecular Biology of the Gene, 4th Ed., W. A. Benjamin, Inc., Menlo Park, Calif. (1987) incorporated herein by reference in its entirety). Typically, vector mediated methodologies involve the introduction of the nucleic acid fragment into a DNA or RNA vector, the clonal amplification of the vector, and the recovery of the amplified nucleic acid fragment. Examples of such methodologies are provided by Cohen et al. (U.S. Pat. No. 4,237,224), Maniatis, T. et al.,

Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory, 1982, each incorporated herein by reference in its entirety.

A number of template dependent processes are available to amplify the target sequences of interest present in a sample. One of the best known amplification methods is the polymerase chain reaction (PCR) which is described in detail in U.S. Patents 4,683,195, 4,683,202 and 4,800,159, and in Innis *et al.*, *PCR Protocols*, Academic Press, Inc., San Diego CA, 1990, each of which is incorporated herein by reference in its entirety. Briefly, in PCR, two primer sequences are prepared which are complementary to regions on opposite complementary strands of the target sequence. An excess of deoxynucleoside triphosphates are added to a reaction mixture along with a DNA polymerase (e.g., *Taq* polymerase). If the target sequence is present in a sample, the primers will bind to the target and the polymerase will cause the primers to be extended along the target sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the target to form reaction products, excess primers will bind to the target and to the reaction products and the process is repeated. Preferably a reverse transcriptase PCR amplification procedure may be performed in order to quantify the amount of mRNA amplified. Polymerase chain reaction methodologies are well known in the art.

Another method for amplification is the ligase chain reaction (referred to as LCR), disclosed in EPA No. 320,308, incorporated herein by reference in its entirety. In LCR, two complementary probe pairs are prepared, and in the presence of the target sequence, each pair will bind to opposite complementary strands of the target such that they abut. In the presence of a ligase, the two probe pairs will link to form a single unit. By temperature cycling, as in PCR, bound ligated units dissociate from the target and then serve as "target sequences" for ligation of excess probe pairs. U.S. Patent 4,883,750, incorporated herein by reference in its entirety, describes an alternative method of amplification similar to LCR for binding probe pairs to a target sequence.

Qbeta Replicase, described in PCT Application No. PCT/US87/00880, incorporated herein by reference in its entirety, may also be used as still another amplification method in the present invention. In this method, a replicative sequence of RNA which has a region complementary to that of a target is added to a sample in the presence of an RNA polymerase. The polymerase will copy the replicative sequence which can then be detected.

An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide 5'-[alpha -thio]triphosphates in one strand of a restriction site (Walker, G. T., *et al.*, *Proc. Natl. Acad, Sci. (U.S.A.)* **1992**, *89*:392-396, incorporated herein by reference in its entirety), may also be useful in the amplification of nucleic acids in the present invention.

Strand Displacement Amplification (SDA) is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, i.e. nick translation. A similar method, called Repair Chain Reaction (RCR) is another method of amplification which may be useful in the present invention and is involves annealing several probes throughout a region targeted for amplification, followed by a repair reaction in which only two of the four bases are present. The other two bases can be added as biotinylated derivatives for easy detection. A similar approach is used in SDA.

Specific sequences can also be detected using a cyclic probe reaction (CPR). In CPR, a probe having a 3' and 5' sequences of non-specific DNA and middle sequence of specific RNA is hybridized to DNA which is present in a sample. Upon hybridization, the reaction is treated with RNaseH, and the products of the probe identified as distinctive products generating a signal which are released after digestion. The original template is annealed to another cycling probe and the reaction is repeated. Thus, CPR involves amplifying a signal generated by hybridization of a probe to a specific expressed nucleic acid.

Still other amplification methods described in GB Application No. 2 202 328, and in PCT Application No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety, may be used in accordance with the present invention. In the former application, "modified" primers are used in a PCR like, template and enzyme dependent synthesis. The primers may be modified by labeling with a capture moiety (e.g., biotin) and/or a detector moiety (e.g., enzyme). In the latter application, an excess of labeled probes are added to a sample. In the presence of the target sequence, the probe binds and is cleaved catalytically. After cleavage, the target sequence is released intact to be bound by excess probe. Cleavage of the labeled probe signals the presence of the target sequence.

Other nucleic acid amplification procedures include transcription-based amplification systems (TAS) (Kwoh D., *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)* **1989**, *86*:1173,

Gingeras T. R., *et al.*, PCT Application WO 88/10315, incorporated herein by reference in their entirety), including nucleic acid sequence based amplification (NASBA) and 3SR. In NASBA, the nucleic acids can be prepared for amplification by standard phenol/chloroform extraction, heat denaturation of a clinical sample, treatment with lysis buffer and minispin columns for isolation of DNA and RNA or guanidinium chloride extraction of RNA. These amplification techniques involve annealing a primer which has sequences specific or complementary to the target nucleic acid sequence the sequence of which is to be increased upon ultrasound. Following polymerization, DNA/RNA hybrids are digested with RNase H while double stranded DNA molecules are heat denatured again. In either case the single stranded DNA is made fully double stranded by addition of second specific primer, followed by polymerization. The double stranded DNA molecules are then multiply transcribed by a polymerase such as T7 or SP6. In an isothermal cyclic reaction, the RNAs are reverse transcribed into double stranded DNA, and transcribed once against with a polymerase such as T7 or SP6. The resulting products, whether truncated or complete, indicate sequences specific for the target nucleic acid sequence.

Davey, C., *et al.*, European Patent Application Publication No. 329,822, incorporated herein by reference in its entirety, disclose a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance with the present invention. The ssRNA is a first template for a first primer oligonucleotide, which is elongated by reverse transcriptase (RNA-dependent DNA polymerase). The RNA is then removed from resulting DNA:RNA duplex by the action of ribonuclease H (RNase H, an RNase specific for RNA in a duplex with either DNA or RNA). The resultant ssDNA is a second template for a second primer, which also includes the sequences of an RNA polymerase promoter (exemplified by T7 RNA polymerase) 5' to its homology to its template. This primer is then extended by DNA polymerase (exemplified by the large "Klenow" fragment of *E. coli* DNA polymerase I), resulting as a double-stranded DNA ("dsDNA") molecule, having a sequence identical to that of the original RNA between the primers and having additionally, at one end, a promoter sequence. This promoter sequence can be used by the appropriate RNA polymerase to make many RNA copies of the DNA. These copies can then re-enter the cycle leading to very swift amplification. With proper choice of enzymes, this amplification can be done isothermally

without addition of enzymes at each cycle. Because of the cyclical nature of this process, the starting sequence can be chosen to be in the form of either DNA or RNA.

Miller, H. I., *et al.*, PCT Application WO 89/06700, incorporated herein by reference in its entirety, disclose a nucleic acid sequence amplification scheme based on the hybridization of a promoter/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic; i.e. new templates are not produced from the resultant RNA transcripts. Other amplification methods include "race" disclosed by Frohman, M. A., In: *PCR Protocols: A Guide to Methods and Applications* **1990**, Academic Press, N.Y.) and "one-sided PCR" (Ohara, O., *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)* **1989**, *86*:5673-5677), all references herein incorporated by reference in their entirety.

Methods based on ligation of two (or more) oligonucleotides in the presence of nucleic acid having the sequence of the resulting "di-oligonucleotide", thereby amplifying the di-oligonucleotide (Wu, D. Y. *et al.*, *Genomics* **1989**, *4*:560, incorporated herein by reference in its entirety), may also be used in the amplification step of the present invention.

Following amplification, the presence or absence of the amplification product may be detected. The amplified product may be sequenced by any method known in the art, including and not limited to the Maxam and Gilbert method, see Sambrook, *supra*. The sequenced amplified product is then compared to a sequence known to be in a target nucleic acid sequence. Alternatively, the nucleic acids may be fragmented into varying sizes of discrete fragments. For example, DNA fragments may be separated according to molecular weight by methods such as and not limited to electrophoresis through an agarose gel matrix. The gels are then analyzed by Southern hybridization. Briefly, DNA in the gel is transferred to a hybridization substrate or matrix such as and not limited to a nitrocellulose sheet and a nylon membrane. A labeled probe is applied to the matrix under selected hybridization conditions so as to hybridize with complementary DNA localized on the matrix. The probe may be of a length capable of forming a stable duplex. The probe may have a size range of about 200 to about 10,000 nucleotides in length, preferably about 200 nucleotides in length, and more preferably about 1462 nucleotides in length. Various labels for visualization or detection are known to those of skill in the art, such as and not limited to fluorescent staining, ethidium bromide staining for example, avidin/biotin, radioactive labeling such as ^{32}P

labeling, and the like. Preferably, the product, such as the PCR product, may be run on an agarose gel and visualized using a stain such as ethidium bromide. See Sambrook *et al.*, *supra*. The matrix may then be analyzed by autoradiography to locate particular fragments which hybridize to the probe.

EXOGENOUS SEQUENCE COMPOSITIONS

For purposes of the present invention, an exogenous nucleic acid sequence may be provided together with an organic halide. An "organic halide" (also sometimes referred to as a halogenated organic compound) denotes a compound which contains at least one carbon atom (or optionally sulfur or selenium atom, such as in the case of SF_6 and SeF_6) and at least one halogen atom selected from the group consisting of fluorine, chlorine, bromine, or iodine. Preferably the halogen is fluorine (i.e., the compound is a fluorinated compound). Most preferably the organic halide is a fluorinated compound which is perfluorinated (that is, fully fluorinated, e.g., a carbon compound wherein all hydrogen atoms directly attached to the carbon atoms have been replaced by fluorine atoms). The perfluorinated organic halide (perfluorinated compound) is preferably a perfluorocarbon or a perfluoroether. The organic halide may be in the form of a gas, a liquid (including a gaseous precursor), or a solid. Preferably the organic halide is a liquid, even more preferably a liquid which is a gaseous precursor that converts to a gas upon administration. Most preferably, the gaseous precursor converts to a gas at the site of (in close or touching proximity to) the cell.

"Gaseous precursor" refers to a liquid or solid which is activated upon attaining a certain temperature or pressure to convert to a gas. A gaseous precursor which is capable of converting to a gas at the site of the cell may increase the efficiency of cellular uptake of compounds, and is therefore preferred.

Ideally, the gaseous precursors are liquid (or solid) at ambient (room) temperature (e.g., 25°C), but will convert to a gas either at physiological temperature (e.g., 37°C) such as upon administration to a patient, or otherwise conveniently at the site of the cell such as upon application of heat (such as, for example, using ultrasound). If heat is applied, it should be done so at a temperature sufficient to convert the gaseous precursor to a gas, but insufficient to harm the cell (e.g., denature the proteins, etc.). Thus, ideally a gaseous precursor becomes a gas at less than about 80°C. Even more ideally, the gaseous precursor becomes a gas at between about 30°C and about 70°C. Most ideally, the gaseous precursor becomes a gas at between about 37°C and less than about 50°C.

A variety of different organic halides may be employed in this invention. Where the organic halide is a carbon based halide compound, the organic halide preferably

contains from 1 to about 30 carbon atoms, more preferably 1 to about 24 carbon atoms, even more preferably 1 to about 12 carbon atoms, still even more preferably about 5 to about 12 carbon atoms, and most preferably about 6 to about 10 carbon atoms. Thus, the number of carbon atoms in the organic halide may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, carbon atoms, and upwards. Sulfur or selenium based halide compounds, such as sulfur hexafluoride and selenium hexafluoride, are also within the scope of the invention and the phrase organic halide as used herein. The organic halides contemplated herein may also, for example, have carbon atoms interrupted by one or more heteroatoms, such as -O- bonds (as in ether compounds) or have other substituents such as amines, etc. Preferred organic halides of the present invention are the perfluorinated organic halides such as perfluorocarbons and perfluoroethers.

Table 1 lists representative organic halides useful in the present invention. Other organic halides suitable for use in the present invention will be readily apparent to one skilled in the art, once armed with the present disclosure. All such organic halides are intended to fall within the scope of the term organic halide, as used herein.

Table 1

Organic Halides

Compound	Boiling Point (^{0}C)
1. Mixed-halogenated Compounds	
1-bromo-nonafluorobutane	43
perfluorooctyliodide	160-161
perfluoroocytlbromide	142
1-chloro-1-fluoro-1-bromomethane	38
1,1,1-trichloro-2,2,2-trifluoroethane	45.7
1,2-dichloro-2,2-difluoroethane	46
1,1-dichloro-1,2-difluoroethane	45
1,2-dichloro-1,1,3-trifluoropropane	50.4
1-bromoperfluorobutane	43
1-bromo-2,4-difluorobenzene	44

2-iodo-1,1,1-trifluoroethane	53
5-bromovaleryl chloride	43
1,3-dichlorotetrafluoroacetone	43

Table 1 (con't.)

bromine pentafluoride	40.3
1-bromo-1,1,2,3,3,3-hexafluoropropane	35.5
2-chloro 1,1,1,4,4,4-hexafluoro-2-butene	33
2-chloropentafluoro-1,3-butadiene	37
iodotrifluoroethylene	30
1,1,2-trifluoro-2-chloroethane	30
1,2-difluorochloroethane	35.5
1,1-difluoro-2-chloroethane	35.1
1,1-dichlorofluoroethane	31.8
heptafluoro-2-iodopropane	39
bromotrifluoroethane	-57.8
chlorotrifluoromethane	-81.5
dichlorodifluoromethane	-29.8
dibromofluoromethane	23
chloropentafluoroethane	-38.7
bromochlorodifluoromethane	-4
dichloro-1,1,2,2-tetrafluoroethane	3.1-3.6

2. Fluorinated Compounds

1,1,1,3,3-pentafluoropentane	40
perfluorotributylamine	178
perfluorotripropylamine	130
3-fluorobenzaldehyde	56
2-fluoro-5-nitrotoluene	53
3-fluorostyrene	40

3,5-difluoroaniline	40
2,2,2-trifluoroethylacrylate	45
3-(trifluoromethoxy)-acetophenone	49
1,1,2,2,3,3,4,4-octafluorobutane	44.8
1,1,1,3,3-pentafluorobutane	40

Table 1 (con't.)

1-fluorobutane	32.5
1,1,2,2,3,3,4,4-octafluorobutane	44.8
1,1,1,3,3-pentafluorobutane	40
perfluoro-4 methylquinolizidine	149
perfluoro-N-methyl-decahydroquinone	150-155
perfluoro-N-methyl-decahydroisoquinone	150-155
perfluoro-N-cyclohexyl-pyrrolidine	145-152
tetradecaperfluoroheptane	76
dodecaperfluorocyclohexane	52

3. Perfluorinated Compounds

a. Perfluorocarbons

perfluoromethane	-129
perfluoroethane	-78.3
perfluoropropane	-36
perfluorobutane	-2
perfluoropentane	29.5
perfluorohexane	59-60
perfluoroheptane	81
perfluorooctane	102
perfluorononane	125
perfluorodecane	~ 143
perfluorododecane	melting pt 75-77

perfluoro-2-methyl-2-pentene	51
perfluorocyclohexane	52
perfluorodecalin	142
perfluorododecalin	---
perfluoropropylene	-28
perfluorocyclobutane	-6
perfluoro-2-butyne	-25

Table 1 (con't.)

perfluoro-2-butene	1.2
perfluorobuta-1,3-diene	6

b. Perfluoroether Compounds

perfluorobutylethyl ether	60
bis(perfluoroisopropyl) ether	54
bis(perfluoropropyl) ether	59
perfluorotetrahydropyran	34
perfluoromethyl tetrahydrofuran	27
perfluoro t-butyl methyl ether	36
perfluoro isobutyl methyl ether	---
perfluoro n-butyl methyl ether	35.4
perfluoro isopropyl ethyl ether	---
perfluoro n-propyl ethyl ether	23.3
perfluoro cyclobutyl methyl ether	---
perfluoro cyclopropyl ethyl ether	---
perfluoro isopropyl methyl ether	36
perfluoro n-propyl methyl ether	---
perflouro diethyl ether	3-4.5
perfluoro cyclopropyl methyl ether	---
perfluoro methyl ethyl ether	-23

perfluoro dimethyl ether	-59

c. Other

sulfur hexafluoride	m.p. -50.5, sublimes -63.8
selenium hexafluoride	m.p. -34.6 sublimes -46.6

Preferred organic halides include 1-bromo-nonafluorobutane, 1,1,1,3,3-pentafluoropentane, perfluorohexane, perfluorocyclohexane, 1-bromo-1,1,2,3,3,3-hexafluoropropane, heptafluoro-2-iodopropane, 1,1,2,2,3,3,4,4-octafluorobutane, 1-fluorobutane, tetradecaperfluoroheptane and dodecaperfluorocylclohexane. Particularly preferred are perfluorohexane (especially n-perfluorohexane) and perfluorocyclohexane. A wide variety of other organic halides useful in the present invention will be readily apparent to those of skill in the art once armed with the present disclosure. Suitable additional organic halides include those, for example, disclosed in Long, Jr. in U.S. Patent Nos. 4,987,154, 4,927,623, and 4,865,836, the disclosures of each of which are hereby incorporated herein by reference in their entirety.

The amount of organic halide employed in the present invention may vary, as one skilled in the art will recognize, once armed with the present disclosure, and may be dependent on such factors as the particular organic halide employed, type and nature of the compound to be delivered, the age, weight, cells or patient (animal) to be treated, the particular diagnostic, therapeutic or other application intended (including the disease state, if any, to be treated). Typically lower amounts are used and then increased until the desired delivery effect is achieved. Representative amounts are set forth in the examples herein. Of course, higher or lower amounts may be employed, as will be recognized by the skilled artisan.

As noted above, the methods of the present invention may, for example, be carried out in the presence of an organic halide, with or without the application of ultrasound, or in the absence of an organic halide but with the application of ultrasound. Where bioactive agents other than nucleotides are employed as the compound to be delivered, generally, for

best results, an organic halide is used, although use of an organic halide in such a situation is not required.

If desired, the composition may further comprise a carrier. The carrier employed may comprise a wide variety of materials. Carriers may include, for example, lipids, polymers, proteins, surfactants, inorganic compounds, metal ions, and the like, alone or in combination with water and/or a solvent, or the carrier may simply comprise water and/or a solvent. The lipids, proteins, and polymers, for example, may be in liquid form or solid form (such as, for example, the form of particles, fibers, sheets, layers, etc.), or may take the form of a vesicle or other stable, organized form, which may include but is not limited to, such forms commonly referred to as, for example, liposomes, micelles, bubbles, microbubbles, microspheres, lipid-, polymer-, and/or protein-coated bubbles, microbubbles and/or microspheres, microballoons, aerogels, hydrogels, clathrates, hexagonal HII phase structures, and the like. The internal void of the vesicle or other stable form may, for example, be filled with a liquid (including, for example, a gaseous precursor), a gas, a solid, or solute material, or any combination thereof, including, for example, the compound to be delivered, the organic halide, and/or any targeting ligand, as desired. Typically, the carrier is provided as an aqueous milieu, such as water, saline (such as phosphate buffered saline), and the like, with or without other carrier components, although other non-aqueous solvents may also be employed, if desired. The carrier may comprise a mixture in the form of an emulsion, suspension, dispersion, solution, and the like. Lipid (including oil) in water emulsions are especially preferred. As indicated above, the carrier may also include buffers.

Thus, "vesicle", as used herein, refers to an entity which is generally characterized by the presence of one or more walls or membranes which form one or more internal voids. Vesicles may be formulated, for example, from stabilizing compounds, such as a lipid, including the various lipids described herein, a polymer, including the various polymers described herein, or a protein, including the various proteins described herein, as well as using other materials that will be readily apparent to one skilled in the art. Other suitable materials include, for example, any of a wide variety of surfactants, inorganic compounds, and other compounds as will be readily apparent to one skilled in the art. Also, as will be apparent to one skilled in the art upon reading the present disclosure, the organic halides may themselves act as suitable carriers, and may in certain embodiments themselves

form vesicles and other organized structures. Thus the use of the organic halides of the invention in combination with a compound to be delivered, without an additional compound to serve as a carrier, is within the scope of the invention. The lipids, polymers, proteins, surfactants, inorganic compounds, and/or other compounds may be natural, synthetic or semi-synthetic. Preferred vesicles are those which comprise walls or membranes formulated from lipids. The walls or membranes may be concentric or otherwise. In the preferred vesicles, the stabilizing compounds may be in the form of a monolayer or bilayer, and the mono- or bilayer stabilizing compounds may be used to form one or more mono- or bilayers. In the case of more than one mono- or bilayer, the mono- or bilayers may be concentric, if desired. Stabilizing compounds may be used to form unilamellar vesicles (comprised of one monolayer or bilayer), oligolamellar vesicles (comprised of about two or about three monolayers or bilayers) or multilamellar vesicles (comprised of more than about three monolayers or bilayers). The walls or membranes of vesicles prepared from lipids, polymers or proteins may be substantially solid (uniform), or they may be porous or semi-porous. The vesicles described herein include such entities commonly referred to as, for example, liposomes, micelles, bubbles, microbubbles, microspheres, lipid-, protein-and/or polymer-coated bubbles, microbubbles and/or microspheres, microballoons, microcapsules, aerogels, clathrate bound vesicles, hexagonal H II phase structures, and the like. The vesicles may also comprise a targeting ligand, if desired.

"Lipid vesicle", "polymer vesicle" and "protein vesicle" refer respectively to vesicles formulated from one or more lipids, polymers and proteins.

"Liposome" refers to a generally spherical or spheroidal cluster or aggregate of amphipathic compounds, including lipid compounds, typically in the form of one or more concentric layers, for example, monolayers or bilayers. They may also be referred to herein as lipid vesicles. The liposomes may be formulated, for example, from ionic lipids and/or non-ionic lipids. Liposomes which are formulated from non-ionic lipids may also be referred to as "niosomes."

"Micelle" refers to colloidal entities formulated from lipids. In certain preferred embodiments, the micelles comprise a monolayer or hexagonal H2 phase configuration. In other preferred embodiments, the micelles may comprise a bilayer configuration.

"Aerogel" refers to generally spherical or spheroidal entities which are characterized by a plurality of small internal voids. The aerogels may be formulated from synthetic or semisynthetic materials (for example, a foam prepared from baking resorcinol and formaldehyde), as well as natural materials, such as polysaccharides or proteins.

"Clathrate" refers to a solid, semi-porous or porous particle which may be associated with vesicles. In preferred form, the clathrates may form a cage-like structure containing cavities which comprise the vesicles. One or more vesicles may be bound to the clathrate. A stabilizing material may, if desired, be associated with the clathrate to promote the association of the vesicle with the clathrate. Suitable materials from which clathrates may be formulated include, for example, porous apatites, such as calcium hydroxyapatite, and precipitates of polymers and metal ions, such as alginic acid precipitated with calcium salts.

"Emulsion" refers to a mixture of two or more generally immiscible liquids and is generally in the form of a colloid. The liquids may be homogeneously or heterogeneously dispersed throughout the emulsion. Alternatively, the liquids may be aggregated in the form of, for example, clusters or layers, including mono- or bilayers.

"Suspension" or "dispersion" refers to a mixture, preferably finely divided, of two or more phases (solid, liquid or gas), such as, for example, liquid in liquid, solid in liquid, liquid in gas, etc.) which can preferably remain stable for extended periods of time.

"Hexagonal H II phase structure" refers to a generally tubular aggregation of lipids, proteins, or polymers (especially lipids) in liquid media, for example, aqueous media, in which any hydrophilic portion(s) generally face inwardly in association with an aqueous liquid environment inside the tube. The hydrophobic portion(s) generally radiate outwardly and the complex assumes the shape of a hexagonal tube. A plurality of tubes is generally packed together in the hexagonal phase structure.

"Biocompatible" refers to materials which are generally not injurious to biological functions and which will not result in any degree of unacceptable toxicity, including allergenic responses and disease states. The compositions of the present invention and/or components thereof are typically biocompatible.

The nucleic acid sequence to be delivered may be administered, if desired, "in combination with" an organic halide, and may further be administered, if desired, "in combination with" a carrier, including a vesicle (or other stable form). "In combination with"

refers to the co-administration of the sequence to be delivered and the organic halide (and/or carrier, if desired). The sequence to be delivered and the organic halide (and/or any carrier) may be combined in any of a variety of different fashions, including simply being placed in admixture with one another. In addition, for example, the nucleic acid sequence to be delivered and/or the organic halide may be embedded, encapsulated, or attached to, or with, one another, as desired (including any and all combinations thereof). The phrase "in admixture" includes solutions, suspensions, emulsions, dispersions, mixtures, etc. The phrase "attached to" or variations thereof, as used herein, denotes being linked in some manner, such as through a covalent or ionic bond or other means of chemical or electrochemical linkage or interaction. The phrase "encapsulated" and variations thereof as used herein refers to a location inside an internal void of a vesicle or other structure. The phrase "embedded within" or variations thereof as used herein signifies a positioning within the wall of a vesicle or other structure. Thus, a nucleic acid sequence, for example, can be positioned variably, such as, for example, entrapped within the internal void of the vesicle, situated on the internal wall of the vesicle, incorporated onto the external surface of the vesicle, and/or enmeshed within the vesicle structure itself. In addition, one or more vesicles may be administered as a cavitator. In such case, the vesicles accompany the administration of a sequence and may serve to enhance the efficiency of ultrasound.

Lipids may be used in the present invention as a carrier. The lipids may be natural, synthetic or semisynthetic (i.e., modified natural). Lipids useful in the invention include, and are not limited to, fatty acids, lysolipids, oils (including safflower, soybean and peanut oil), phosphatidylcholine with both saturated and unsaturated lipids including phosphatidylcholine; dioleoylphosphatidylcholine; dimyristoylphosphatidylcholine; dipentadecanoylphosphatidylcholine, dilauroylphosphatidylcholine, dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine; distearoylphosphatidylcholine; phosphatidylethanolamines such as dioleoylphosphatidylethanolamine; phosphatidylserine; phosphatidylglycerol; phosphatidylinositol, sphingolipids such as sphingomyelin; glycolipids such as ganglioside GM1 and GM2; glucolipids; sulfatides; glycosphingolipids; phosphatidic acid; palmitic acid; stearic acid; arachidonic acid; oleic acid; lipids bearing polymers such as polyethyleneglycol, chitin, hyaluronic acid or polyvinylpyrrolidone; lipids bearing sulfonated mono-, di-, oligo- or polysaccharides; cholesterol, cholesterol sulfate and cholesterol

hemisuccinate; tocopherol hemisuccinate, lipids with ether and ester-linked fatty acids, polymerized lipids (a wide variety of which are known in the art), diacetyl phosphate, stearylamine, cardiolipin, phospholipids with short chain fatty acids of about 6 to about 8 carbons in length, synthetic phospholipids with asymmetric acyl chains (e.g., with one acyl chain of about 6 carbons and another acyl chain of about 12 carbons), 6-(5-cholesten-3β-yloxy)-1-thio-b-D-galactopyranoside, digalactosyldiglyceride, 6-(5-cholesten-3β-yloxy)hexyl-6-amino-6-deoxy-1-thio-β-D-galactopyranoside, 6-(5-cholesten-3β-yloxy)hexyl-6-amino-6-deoxyl-1-thio-α-D-mannopyranoside, 12-(((7'-diethylamino-coumarin-3-yl)carbonyl)methylamino)-octadecanoic acid; N-[12-(((7'-diethylaminocoumarin-3-yl)carbonyl)methyl-amino) octadecanoyl]-2-aminopalmitic acid; (cholesteryl)4'-trimethyl-ammonio)butanoate; N-succinyldioleoylphosphatidylethanolamine; 1,2-dioleoyl-sn-glycerol; 1,2-dipalmitoyl-sn-3-succinyl-glycerol; 1,3-dipalmitoyl-2-succinylglycerol; 1-hexadecyl-2-palmitoyl-glycerophosphoethanolamine and palmitoylhomocysteine, and/or combinations thereof. Vesicles or other structures may be formed of the lipids, either as monolayers, bilayers, or multilayers and may or may not have a further coating.

The preferred lipid carrier may be in the form of a monolayer or bilayer, and the mono- or bilayer may be used to form one or more mono- or bilayers. In the case of more than one mono- or bilayer, the mono- or bilayers may be concentric. The carrier may form a unilamellar vesicle (comprised of one monolayer or bilayer), an oligolamellar vesicle (comprised of about two or about three monolayers or bilayers) or a multilamellar vesicle (comprised of more than about three monolayers or bilayers). The walls or membranes of a vesicle may be substantially solid (uniform), or they may be porous or semi-porous.

Lipids bearing hydrophilic polymers such as polyethyleneglycol (PEG), including and not limited to PEG 2,000 MW, 5,000 MW, and PEG 8,000 MW, are particularly useful for improving the stability and size distribution of organic halide-containing composition. Dipalmitoylphosphatidylcholine (DPPC) may be useful in the present invention at about 70% to about 90%, dipalmitoylphosphatidylethanolamine-polyethylene glycol 5000 (DPPE-PEG 5000) may be useful at about 0% to about 20% and dipalmitoylphosphatidic acid (DPPA) may be useful at about 0% to about 20% (all percentages being in mole percent molecular weight). A preferred product which is highly

useful as a carrier in the present invention contains about 82 mole percent DPPC, about 8 mole percent DPPE-PEG 5,000 MW and about 10 mole percent DPPA. Various different mole ratios of PEGylated lipid are also useful.

Additionally lipid moieties capable of polymerization are embraced in the invention as coatings for the vesicles. Examples of these include, but are not limited to, alkenyl and alkynyl moieties, such as oleyl and linoleyl groups, diacetylene, acryloyl and methacryloyl groups with or without polar groups to enhance water solubility, cyanoacrylate esters optionally carrying lipophilic esterifying groups or the compounds illustrated as A and B, below. A number of such compounds are described, for example, in Klaveness et al., U.S. Patent No. 5,536,490. The disclosures of Klaveness et al., U.S. Patent No. 5,536,490, are hereby incorporated herein by reference in their entirety.

Fluorinated or perfluorinated lipids may also be used in this invention, either as the organic halide component or as an additional carrier material. Examples of suitable fluorinated lipids include but are not limited to compounds of the formula

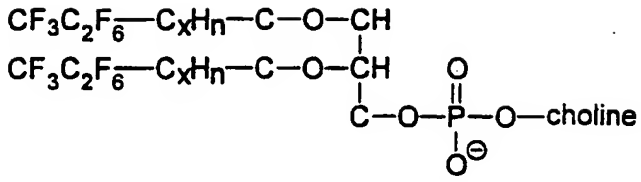
$$C_nF_{2n+1}(CH_2)_mC(O)OOP(OO^-)O(CH_2)_W\ N^+(CH_3)_3C_nF_{2n+1}(CH_2)_mC(O)O$$

wherein: m is 0 to about 18, n is 1 to about 12; and w is 1 to about 8. Examples of and methods for the synthesis of these, as well as other fluorinated lipids useful in the present invention, are set forth in U.S. application Serial No. 08/465,868, filed June 6, 1995, Reiss et al. U.S. Patent No. 5,344,930, Frezard, F., *et al., Biochem Biophys Acta* **1994**, *1192*:61-70, and Frezard, F., *et al., Art. Cells Blood Subs and Immob Biotech.* **1994**, *22*:1403-1408, the disclosures of each of which are incorporated herein by reference in their entirety. One specific example of a difluoroacyl glycerylphosphatidylcholine, nonafluorinated diacyl glycerylphosphatidylcholine, is represented by compound A, below. Those skilled in the art will appreciate that analogous fluorinated derivatives of other common phospholipids (diacylphosphatidyl serine, diacylphosphatidyl ethanolamine, diacylphosphatidyl glycerol, diacylphosphatidyl glycerol, etc.) as well as fluorinated derivatives of fatty acyl esters and free fatty acids may also function in accordance with the scope of the invention. Additionally lipid based and fluorinated (including perfluorinated) surfactants such as may be used as carriers in the present invention.

A wide variety of such fluorinated compounds may be employed, including, for example, the class of compounds which are commercially available as ZONYL® fluorosurfactants (the DuPont Company, Wilmington, DE), including the ZONYL® phosphate salts and ZONYL® sulfate salts, which are fluorosurfactants having terminal phosphate or sulfate groups. Representative compounds are disclosed, for example, in U.S. Patent No. 5,276,145, the disclosures of which are hereby incorporated herein by reference in their entirety. Suitable ZONYL® surfactants also include, for example, ZONYL® surfactants identified as Telomer B, including Telomer B surfactants which are pegylated (i.e., have at least one polyethylene glycol group attached thereto), also known as PEG-Telomer B, available from the DuPont Company. Most preferred are such pegylated fluorosurfactants.

Suitable polymerizable and/or fluorinated compounds include:

A



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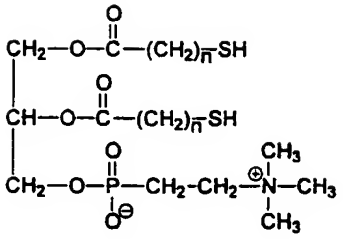

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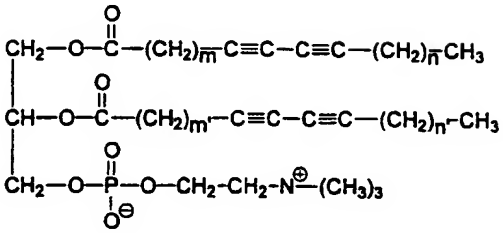

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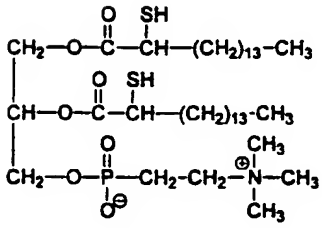

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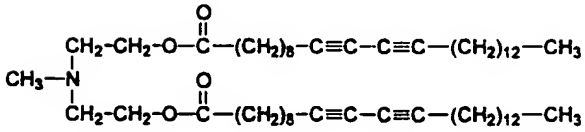

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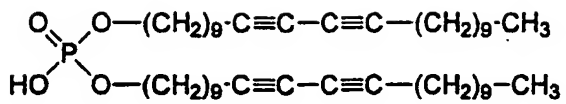

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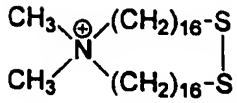

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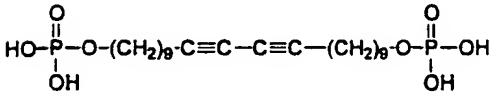
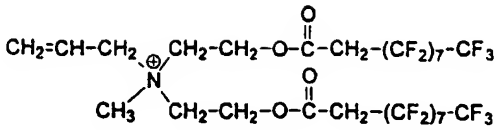


I



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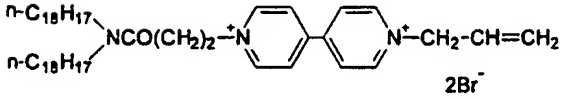

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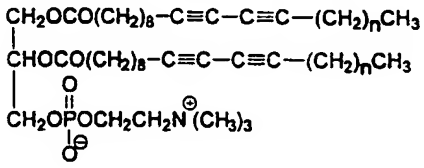

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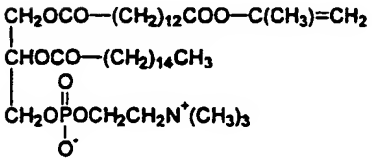

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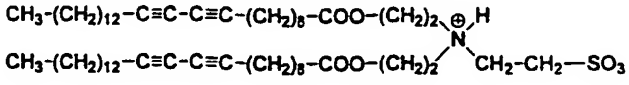

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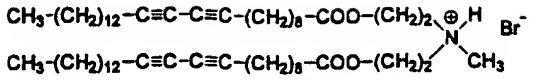

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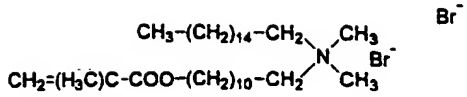

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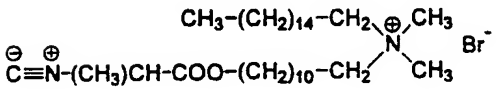

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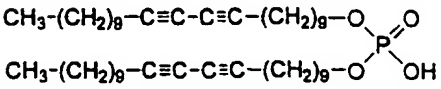

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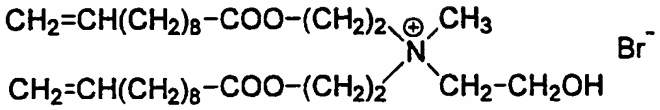

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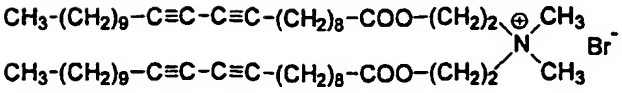

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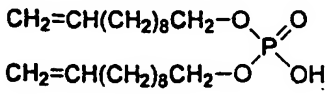

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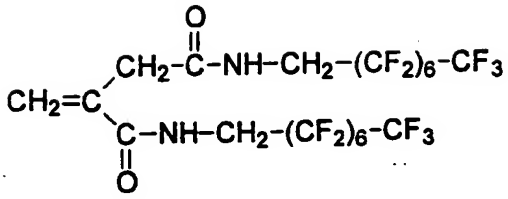

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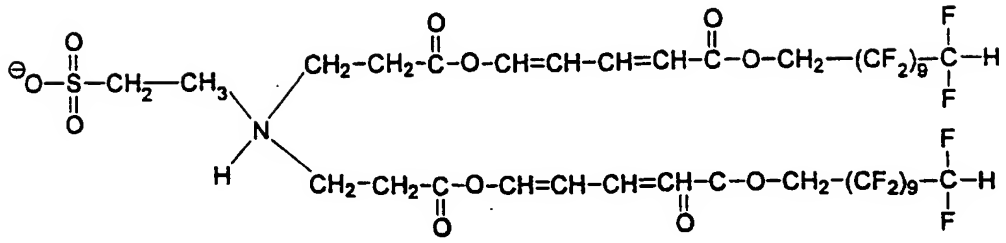

In formula A, above, preferably x is an integer from about 8 to about 18, and n is 2x. Most preferably x is 12 and n is 24.

Cationic lipids and other derivatized lipids and lipid mixtures also may be useful as carriers in the present invention. Suitable cationic lipids include dimyristyl oxypropyl-3-dimethylhydroxy ethylammonium bromide (DMRIE), dilauryl oxypropyl-3-dimethylhydroxy ethylammonium bromide (DLRIE), N-[1-(2,3-dioleoyloxyl)propal]-n,n,n-trimethylammonium sulfate (DOTAP), dioleoylphosphatidylethanolamine (DOPE), dipalmitoylethylphosphatidylcholine (DPEPC), dioleoylphosphatidylcholine (DOPC), polylysine, lipopolylysine, didoceyl methylammonium bromide(DDAB), 2,3-dioleoyloxy-N-[2-(sperminecarboxamidoethyl]-N,N-di-methyl-1-propanaminium trifluoroacetate (DOSPA), cetyltrimethylammonium bromide (CTAB), lysyl-PE, 3,β-[N,(N',N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-Cholesterol, also known as DC-Chol), (-alanyl cholesterol, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride(DOTMA), dipalmitoylphosphatidylethanolamine-5-carboxyspermylamide (DPPES), dicaproylphosphatidylethanolamine (DCPE), 4-dimethylaminopyridine (DMAP),

dimyristoylphosphatidylethanolamine (DMPE), dioleoylethylphosphocholine (DOEPC), dioctadecylamidoglycyl spermidine (DOGS), N-[1-(2,3-dioleoyloxy)propyl]-N-[1-(2-hydroxyethyl)]-N,N-dimethylammonium iodide (DOHME), Lipofectin (DOTMA + DOPE, Life Technologies, Inc., Gaithersburg, MD), Lipofectamine (DOSPA + DOPE, Life Technologies, Inc., Gaithersburg, MD), Transfectace (Life Technologies, Inc., Gaithersburg, MD), Transfectam (Promega Ltd., Madison, WI), Cytofectin (Life Technologies Inc., Gaithersburg, MD). Other representative cationic lipids include but are not limited to phosphatidylethanolamine, phospatidylcholine, glycero-3-ethylphosphatidylcholine and fatty acyl esters thereof, di- and trimethyl ammonium propane, di- and tri-ethylammonium propane and fatty acyl esters thereof. A preferred derivative from this group is N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA). Additionally, a wide array of synthetic cationic lipids function as compounds useful in the invention. These include common natural lipids derivatized to contain one or more basic functional groups. Examples of lipids which may be so modified include but are not limited to dimethyldioctadecylammonium bromide, sphingolipids, sphingomyelin, lysolipids, glycolipids such as ganglioside GM1, sulfatides, glycosphingolipids, cholesterol and cholesterol esters and salts, N-succinyldioleoylphosphatidylethanolamine, 1,2,-dioleoyl-sn-glycerol, 1,3-dipalmitoyl-2-succinylglycerol, 1,2-dipalmitoyl-sn-3-succinylglycerol, 1-hexadecyl-2-palmitoylglycerophosphatidyl-ethanolamine and palmitoylhomocystiene.

Other synthesized cationic lipids that are useful in the present invention are those disclosed in pending U.S. patent application Serial No. 08/391,938, filed February 2, 1995, and include, for example, N,N'-Bis (dodecyaminocarbonylmethylene)-N,N'bis ((-N,N,N-trimethylammoniumethyl- aminocarbonylmethylene)ethylenediamine tetraiodide; N,N''-Bis (hexadecylaminocarbonylmethylene)-N,N',N''-tris ((-N,N,N-trimethylammonium-ethylaminocarbonylmethylenedi-ethylenetriamine hexaiodide; N,N'-Bis (dodecylaminocarbonylmethylene)-N,N^{n}-bis((-N,N,N-trimethylammoniumethylamino-carbonylmethylene)cyclohexylene-1,4-diamine tetraiodide; 1,1,7,7-tetra-((-N,N,N,N-tetramethylammoniumethylaminocarbonylmethylene)-3-hexadecyl-aminocarbonyl-methylene-1,3,7-triaazaheptane heptaiodide; and N,N,N'N'-tetra((-N,N,N-

trimethylammoniumethylaminocarbonylmethylene)-N'-(1,2-dioleoylglycero-3-phosphoeth anolaminocarbonylmethylene) diethylenetriamine tetraiodide. Those of skill in the art will recognize that countless other natural and synthetic variants carrying positive charged moieties will also function in the invention.

Also useful as carriers in the present invention are a wide variety of surfactants (i.e., surface-active agents), including polyoxyalkylene fatty acid esters (such as polyoxyethylene fatty acid esters), polyoxyalkylene fatty alcohols (such as polyoxyethylene fatty alcohols), polyoxyalkylene fatty alcohol ethers (such as polyoxyethylene fatty alcohol ethers), polyoxyalkylene sorbitan fatty esters (such as, for example, the class of compounds referred to as TWEEN™, commercially available from ICI Americas, Inc., Wilmington, DE), including poly(oxyethylene)poly(oxypropylene) copolymers (such as Pluronics), polysorbates (such as Tween20, Tween40, and Tween80), polyoxyethylene alcohols (such as Brij), and plasmalogens, the term applied to a number of a group of phospholipids present in platelets that liberate higher fatty aldehydes, e.g. palmital, on hydrolysis and may be related to the specialized function of platelets in blood coagulation and plasmalogens are also present in cell membranes of muscle and the myelin sheath of nerve fibers.

In the preferred embodiment of the invention the organic halide is incorporated into the core of a vesicle which vesicle carrier is also used to complex the nucleic acid sequence to be delivered.

A wide variety of oils may be preferably employed as carriers in the present invention including, but not limited to, safflower, soybean, and peanut oil. The composition may take the form of an oil in water emulsion if desired.

The most preferred carrier is a cationic lipid (including a cationic liposome), particularly as employed in an aqueous milieu. A preferred cationic lipid is DPEPC in admixture with the neutral fusogenic lipid dioleoylphosphatidylethanolamine (DOPE). A preferred ratio of lipid to organic halide is 5:1 w/w. A preferred embodiment is to formulate the lipid or polymer as an organic halide-filled microsphere, such as a microsphere formed with the lipids dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylethanolamine coupled to polyethylene glycol 5000 (DPPE-PEG5000), and dipalmitoylphosphatidic acid (DPPA). DPPC:DPPE-PEG5000:DPPA may be combined in a ratio of about 82%:8%:10% (mole %) or 83%:8%:5%. DPPE-PEG5000 is comprised of DPPE and PEG5000 in a ratio

of about 20%:80% (weight %). PEG5000 refers to PEG having an average molecular weight of about 5000.

Proteins (including peptides) useful as carriers in accordance with the present invention include molecules comprising, and preferably consisting essentially of, α-amino acids in peptide linkages. A wide variety of proteins may be employed as carriers in the present invention, including natural, synthetic, or semi-synthetic proteins. Included within the term "protein" are globular proteins, such as albumins, globulins and histones, and fibrous proteins such as collagens, elastins and keratins. Also included are "compound proteins", wherein a protein molecule is united with a nonprotein molecule, such as nucleproteins, mucoproteins, lipoproteins, and metalloproteins. Preferable proteinaceous macromolecules include for example, albumin, collagen, polyarginine, polylysine, polyhistidine, γ-globulin and β-globulin, with albumin, polyarginine, polylysine, and polyhistidine being more preferred. Fluorinated peptides and synthetic pseudopeptides are also useful as carriers. Fluorinated peptides useful in the present invention include those described in Lohrmann, U.S. Patent No. 5,562,892, the disclosures of which are hereby incorporated herein by reference in their entirety. Cationic peptides may also be usefully employed as carriers in the present invention. Various peptides suitable for use in the present invention will be apparent to one skilled in the art based on the present disclosure.

The methods of the present invention may also involve vesicles or other organized stable form formulated from proteins, peptides and/or derivatives thereof. Vesicles which are formulated from proteins and which would be suitable for use in the methods of the present invention are described, for example, in Feinstein, U.S. Patent Nos. 4,572,203, 4,718,433, and 4,774,958, and Cerny et al., U.S. Patent No. 4,957,656, all of the disclosures of each of which are hereby incorporated by reference in their entirety. Other protein-based vesicles, in addition to those described in the aforementioned patents, would be apparent to one of ordinary skill in the art, once armed with the present disclosure.

Included among the methods described in the aforementioned patents for the preparation of protein-based vesicles are methods which involve sonicating a solution of a protein. In preferred form, the starting material may be an aqueous solution of a heat-denaturable, water-soluble biocompatible protein. The encapsulating protein is preferably heat-sensitive so that it can be partially insolubilized by heating during sonication. Suitable

heat-sensitive proteins include, for example, albumin, hemoglobin, collagen, and the like. Preferably, the protein is a human protein, with human serum albumin (HSA) being more preferred. HSA is available commercially as a sterile 5% aqueous solution, which is suitable for use in the preparation of protein-based vesicles. Of course, as would be apparent to one of ordinary skill in the art, other concentrations of albumin, as well as other proteins which are heat-denaturable, can be used to prepare the vesicles. Generally speaking, the concentration of HSA can vary and may range from about 0.1 to about 25% by weight, and all combinations and subcombinations of ranges therein. It may be preferable, in connection with certain methods for the preparation of protein-based vesicles, to utilize the protein in the form of a dilute aqueous solution. For albumin, it may be preferred to utilize an aqueous solution containing from about 0.5 to about 7.5% by weight albumin, with concentrations of less than about 5% by weight being preferred, for example, from about 0.5 to about 3% by weight.

The protein-based vesicles may be prepared using equipment which is commercially available. For example, in connection with a feed preparation operation as disclosed, for example, in Cerny, et al., U.S. Patent No. 4,957,656, stainless steel tanks which are commercially available from Walker Stainless Equipment Co. (New Lisbon, WI), and process filters which are commercially available from Millipore (Bedford, MA), may be utilized.

The sonication operation may utilize both a heat exchanger and a flow through sonicating vessel, in series. Heat exchanger equipment of this type may be obtained from ITT Standard (Buffalo, NY). The heat exchanger maintains operating temperature for the sonication process, with temperature controls ranging from about 65°C to about 80°C, depending on the makeup of the media. The vibration frequency of the sonication equipment may vary over a wide range, for example, from about 5 to about 40 kilohertz (kHz), with a majority of the commercially available sonicators operating at about 10 or 20 kHz. Suitable sonicating equipment include, for example, a Sonics & Materials Vibra-Cell, equipped with a flat-tipped sonicator horn, commercially available from Sonics & Materials, Inc. (Danbury, CT). The power applied to the sonicator horn can be varied over power settings scaled from 1 to 10 by the manufacturer, as with Sonics & Materials Vibra-Cell Model VL1500. An intermediate power setting, for example, from 5 to 9, can be used. It is preferred that the

vibrational frequency and the power supplied be sufficient to produce cavitation in the liquid being sonicated. Feed flow rates may range from about 50 mL/min to about 1000 mL/min, and all combinations and subcombinations of ranges therein. Residence times in the sonication vessel can range from about 1 second to about 4 minutes, and gaseous fluid addition rates may range from about 10 cubic centimeters (cc) per minute to about 100 cc/min, or 5% to 25% of the feed flow rate, and all combinations and subcombinations of ranges therein.

It may be preferable to carry out the sonication in such a manner to produce foaming, and especially intense foaming, of the solution. Generally speaking, intense foaming and aerosolating are important for obtaining a contrast agent having enhanced concentration and stability. To promote foaming, the power input to the sonicator horn may be increased, and the process may be operated under mild pressure, for example, about 1 to about 5 psi. Foaming may be easily detected by the cloudy appearance of the solution, and by the foam produced.

Such sonication methods may also be employed to prepare lipid-based or other types of carriers as will be apparent to the skilled artisan.

Suitable methods for the preparation of protein-based vesicles may involve physically or chemically altering the protein or protein derivative in aqueous solution to denature or fix the material. For example, protein-based vesicles may be prepared from a 5% aqueous solution of HSA by heating after formation or during formation of the contrast agent via sonication. Chemical alteration may involve chemically denaturing or fixing by binding the protein with a difunctional aldehyde, such as glutaraldehyde. For example, the vesicles may be reacted with 0.25 grams of 50% aqueous glutaraldehyde per gram of protein at pH 4.5 for 6 hours. The unreacted glutaraldehyde may then be washed away from the protein.

The carriers may also be formulated with polymers, natural, synthetic, or semisynthetic. A wide variety of polymers may be utilized as carriers in the present invention, including synthetic polymers including polyethylenes (such as, for example, polyethylene glycol), polyoxyethylenes (such as, for example, polyoxyethylene glycol), polypropylenes (such as, for example, polypropylene glycol), pluronic acids and alcohols, polyvinyls (such as, for example, polyvinyl alcohol), and polyvinylpyrrolidone. Exemplary natural polymers suitable for use in the present invention include polysaccharides.

Polysaccharides include, for example, arabinans, fructans, fucans, galactans, galacturonans, glucans, mannans, xylans (such as, for example, inulin), levan, fucoidan, carrageenan, galactocarolose, pectin (including high methoxy pectin and low methoxy pectin; with low methoxy pectin denoting pectin in which less than 40% of the carboxylic acid groups are esterified and/or amidated, and high methoxy pectin denoting pectin in which 40% or more of the carboxylic acid groups are esterified and/or amidated), pectic acid, amylose, pullulan, glycogen, amylopectin, cellulose, carboxylmethylcellulose, hydroxypropyl methylcellulose, dextran, pustulan, chitin, agarose, keratan, chondroitin, dermatan, hyaluronic acid and alginic acid, and various other homopolymers or heteropolymers such as those containing one or more of the following aldoses, ketoses, acids or amines: erythrose, threose, ribose, arabinose, xylose, lyxose, allose, altrose, glucose, mannose, gulose, idose, galactose, talose, erythrulose, ribulose, xylulose, psicose, fructose, sorbose, tagatose, glucuronic acid, gluconic acid, glucaric acid, galacturonic acid, mannuronic acid, guluronic acid, glucosamine, galactosamine and neuraminic acid. It is recognized that some polymers may be prepared by chemically modifying naturally occurring polymers. Such chemically modified natural polymers also referred to as semisynthetic polymers. The polymers employed may also comprise fluorinated polymers, including those described in Lohrmann, U.S. Patent No. 5,562,892, the disclosures of which are hereby incorporated herein by reference in their entirety. Furthermore, the polymers may be in the form of vesicles, such as for example, those described in Unger, U.S. Patent No. 5,205,290, the disclosures of which are hereby incorporated herein by reference in their entirety. As used herein, the term "polymer" denotes molecules formed from the chemical union of two or more repeating units, and include dimers, trimers, and oligomers. In preferred form, the term "polymer" refers to molecules which comprise 10 or more repeating units.

Metal ions may also be employed as carriers in the present invention. Suitable metal ions include calcium ions, magnesium ions, zinc ions, and the like, as well as a wide variety of inorganic compounds. Other suitable metal ions as well as other suitable inorganic compounds will be readily apparent to those skilled in the art once armed with the present invention.

Other useful agents that may be employed in the carrier of the present invention include osmotic agents, anti-microbials, viscosity raising agents, suspending agents, humectants and anti-humectants, depending upon the particular formulation desired.

One or more emulsifying or stabilizing agents may also be employed as or be included in the carrier. These agents help to maintain the size of any discrete units (e.g., liquid droplets, particles, gas bubbles, etc.) of the organic halide and/or compounds to be delivered that may have formed the composition. The size of these discrete units will generally affect the size of any resultant gas bubbles that may form from any gaseous precursors. The emulsifying and stabilizing agents also may be used to generally coat or stabilize the organic halides, compounds to be delivered, etc. Stabilization is desirable to maximize the intracellular delivery effect. Although stabilization is preferred, this is not an absolute requirement. Because any gas resulting from organic halide gaseous precursors is more stable than air, they may still be designed to provide useful delivery means; for example, they pass through the pulmonary circulation following peripheral venous injection, even when not specifically stabilized by one or more coating or emulsifying agents. One or more coating or stabilizing agents is preferred however, as are flexible stabilizing materials. Also, it should be noted that compositions stabilized by polysaccharides, gangliosides, and polymers are generally more effective than those stabilized by albumin and other proteins. Also, liposomes prepared using aliphatic compounds are preferred, since microspheres stabilized with these compounds are much more flexible and stable to pressure changes.

The carrier of the invention may also comprise a wide variety of viscosity modifiers, including and not limited to carbohydrates and their phosphorylated and sulfonated derivatives; polyethers, preferably with molecular weight ranges between 400 and 8000; di- and trihydroxy alkanes and their polymers, preferably with molecular weight ranges between 800 and 8000. Glycerol propylene glycol, polyethylene glycol, polyvinyl pyrrolidone, and polyvinyl alcohol may also be useful as carriers or stabilizers in the present invention. Particles which are porous or semi-solid such as hydroxyapatite, metal oxides and coprecipitates of gels, e.g., hyaluronic acid with calcium may be used and may formulate a center or nidus to stabilize compositions of the invention.

Emulsifying and/or solubilizing agents may also be used in a carrier, particularly in conjunction with lipids or liposomes. Such agents include and are not limited

to, acacia, cholesterol, diethanolamine, glyceryl monostearate, lanolin alcohols, lecithin, mono- and di-glycerides, mono-ethanolamine, oleic acid, oleyl alcohol, poloxamer, polyoxyethylene 50 stearate, polyoxyl 35 castor oil, polyoxyl 10 oleyl ether, polyoxyl 20 cetostearyl ether, polyoxyl 40 stearate, polysorbate 20, polysorbate 40, polysorbate 60, polysorbate 80, propylene glycol diacetate, propylene glycol monostearate, sodium lauryl sulfate, sodium stearate, sorbitan, sorbitan mono-laurate, sorbitan mono-oleate, sorbitan mono-palmitate, sorbitan monostearate, stearic acid, trolamine, and emulsifying wax. All lipids with perfluoro fatty acids as a component of the lipid in lieu of the saturated or unsaturated hydrocarbon fatty acids found in lipids of plant or animal origin may be used. Suspending and/or viscosity-increasing agents that may be particularly useful with lipid or liposome solutions include but are not limited to, acacia, agar, alginic acid, aluminum mono-stearate, bentonite, magma, carbomer 934P, carboxymethylcellulose, calcium and sodium and sodium 12, glycerol, carrageenan, cellulose, dextrin, gelatin, guar gum, hydroxyethyl cellulose, hydroxypropyl methylcellulose, magnesium aluminum silicate, methylcellulose, pectin, polyethylene oxide, polyvinyl alcohol, povidone, propylene glycol, alginate, silicon dioxide, sodium alginate, tragacanth, and xanthum gum. A preferred product of the present invention incorporates lipid as a mixed solvent system in a ratio of 8:1:1 or 9:1:1 normal saline:glycerol:propylene glycol.

The amount of carrier material employed in connection with the subject invention may vary, as one skilled in the art will recognize upon being placed in possession of the subject disclosure, and may be dependent on such factors as the particular carrier used, the type and nature of the compound to be delivered, the age, weight, cells or patient (animal) to be treated, the particular diagnostic, therapeutic or other application intended (including the disease state, if any, to be treated), and the organic halide (if any) used. Generally, smaller amounts of carrier are employed, and increased until the desired delivery result is obtained. Representative amounts are set forth in the examples herein. Of course, higher or lower amounts may be employed, as will be recognized by the skilled artisan.

METHODS OF MAKING COMPOSITIONS COMPRISING EXOGENOUS SEQUENCES

A wide variety of different methods may be used to mix the organic halide, sequence to be delivered, and/or carrier, and incorporate the sequence to be delivered with or into any organic halide and/or carrier. Methods include shaking by hand, vortexing, mechanical shaking (e.g. with an Espe CapMix, Espe Medizin-Dental GMBH, Seefeld, Germany), extruder (e.g. with a Lipex Biomembranes Extruder Device, Vancouver, B.C., Canada), microemulsification (e.g. with a Microfluidizer, Microfluidics Corp., Newton, MA), mixing with static in line mixers (Cole-Parmer Instrument Co., Vernon Hills, IL), spray drying (e.g. with a Bucchi spray dryer, Brinkmann Ind., Inc., Westbury, MA), mechanical stirring/mixing (e.g. with a Silverson Mixer, Silverson Machines, Ltd., Waterside Chesham Bucks, England) and sonication. In general it is desirable to mix the carrier (e.g. lipids such as DPEPC and DOPE) together with the organic halide prior to adding the compound to be delivered (e.g., DNA). After adding the DNA, a carrier and organic halide association will form with the DNA. If desired, additional mixing may then be performed by one of the above techniques. In some other situations, e.g. calcium precipitation, the DNA, organic halide, and cations may be added together with one or more stabilizing agents to form the precipitates of DNA/carrier/organic halide in a single step process. Again, one of a variety of mixing techniques as described above may be employed to decrease the size of the resultant particles.

The carriers may be combined with the sequence to be delivered and the organic halide in varying amounts and percentages, as will be understood by those skilled in the art once armed with the present disclosure. Typically, smaller amounts of all compositional components are employed, and increased selectively in increments until the desired delivery effect is achieved. Generally, when the sequence to be delivered is employed with a carrier, the ratio of organic halide and any carrier to the sequence to be delivered may be from about 6 to about 1, to about 1 to about 6, and variations therebetween. Preferably, the carrier to sequence to be delivered ratio is about 6 to about 1. Representative ratios are provided by the examples herein. Of course, other ratios can be suitably employed over a wide variety of ranges as desired, as will be recognized by the skilled artisan, and all such ratios are within the scope of the present invention.

The resulting composition may be stored as a lyophilized, or freeze dried, material for inhalation or hydration prior to use or as a preformed suspension. Cryopreservatives known to skilled artisans once armed with the present disclosure may be used in the lyophilized form of the composition. To prevent agglutination or fusion of vesicles as a result of lyophilization, it may be useful to include additives which prevent such fusion or agglutination from occurring. Additives which may be useful include sorbitol, mannitol, sodium chloride, glucose, trehalose, polyvinylpyrrolidone and poly(ethylene glycol) (PEG), for example, PEG polymers having a molecular weight of from about 400 to about 10,000, with PEG polymers having molecular weights of about 1000, 3000 (such as PEG3350) and 5000 being preferred. These and other additives are described in the literature, such as in the U.S. Pharmacopeia, USP XXII, NF XVII, The United States Pharmacopeia, The National Formulary, United States Pharmacopeial Convention Inc., 12601 Twinbrook Parkway, Rockville, MD 20852, the disclosures of which are hereby incorporated herein by reference in their entirety. Lyophilized preparations generally have the advantage of greater shelf life. As noted above, if desired, the lyophilized composition may be (and preferably is) rehydrated prior to use.

The route of administration varies depending upon the intended application. For cell culture applications, the composition is typically contacted with the cells by, for example, adding it to the cell culture media or applying it directly to the cells. Advantages of this invention for transfection in cell culture media include high activity in serum containing media and a single step transfection process with higher efficiency transfection than in other more complicated systems. Indeed, the present invention makes it possible to obtain gene expression in cells in which transfection was otherwise impossible or extremely difficult. For *in vivo* administration the composition may simply be injected, such as intravenously, intravascularly, intralymphatically, parenterally, subcutaneously, intramuscularly, intranasally, intrarectally, intraperitoneally, interstitially, into the airways via nebulizer, hyperbarically, orally, topically, or intratumorly, or otherwise administered.

One or more targeting ligands may be incorporated into the carrier to facilitate uptake by selected cells. Targeting ligands include, for example, peptides, antibodies, antibody fragments, glycoproteins, carbohydrates, etc. Preferably, the targeting ligand is covalently attached to the carrier, e.g., to a lipid. Preferably the targeting ligand is attached

to a linker which is attached to the surface of the carrier. Preferred linkers are polymers, for example, bifunctional PEG having a molecular weight of about 1,000 to about 10,000, most preferably 5,000. Generally, the targeting ligand is incorporated into the carrier from about 0.1 mole % to about 25 mole %, preferably about 1 mole % to about 10 mole %.

In this regard, the composition may be targeted to coated pits of selected cells and taken up into endosomes via a process of receptor mediated endocytosis. Ultrasound energy may be applied to the target tissue to increase nucleic acid synthesis. For inhalation the composition may be inhaled via a nebulizer or via an inhaler. Also, oral or rectal routes may be utilized to administer these composition. Transcutaneous application may be accomplished by the use of penetration enhancing agents with or without the application of sonophoresis (e.g. low frequency sound in range of 10 to 100 Khz) or iontophoresis. Also interstitial (e.g. intratumoral) and subcutaneous injection may be performed to administer the composition.

Also the invention may be practiced with gene gun techniques or electroporation, or in combination with other transfection techniques known in the art. In either case, ultrasound may be applied to the cells before, after, and/or simultaneously with the gene gun or electroporation procedure. The electric fields of electroporation may also be pulsed in tandem with the ultrasound energy to further increase the efficacy of transfection.

Ultrasound is employed at a frequency and energy level sufficient to assist in increasing the synthesis of nucleic acids in the cell. Where organic halide gaseous precursors are employed, the ultrasound may be applied at a frequency and energy level sufficient to convert the organic halide gaseous precursor to a gas. For example, the present invention comprises administering a nucleic acid sequence to a cell and applying ultrasound to the cell for a time effective to increase synthesis of the nucleic acid sequence. Enhanced delivery of exogenous sequences also results. Ultrasound is carried out at a frequency, energy level, and duty cycle for a therapeutically effective time in which to induce delivery of the nucleotide sequence. Suitable frequencies, energy levels and duty cycles are disclosed herein, and other ranges will be readily apparent to one skilled in the art once armed with the present disclosure.

METHODS OF USING THE PRESENT INVENTION

The methods of the present invention increase the synthesis of nucleic acid sequences coding for a variety of proteins, and antisense sequences which block synthesis of a variety of nucleic acids. As a result, a number of diseases may be treated with the methods of the present invention. In addition, the methods of the present invention may be practiced *in vivo*, *ex vivo*, and *in vitro*.

Administration of a nucleic acid sequence by a microsphere utilizes a nucleic acid sequence attached to a microsphere in various positions relative to the microsphere as set forth above. While not intending to be bound by any particular theory or theories of operation, the microsphere approach is believed to rely on the fusion of the nucleic acid sequence containing microsphere with the plasma membrane of the host cell. The nucleic acid sequence subsequently traverses the cytoplasm and enters the nucleus. The use of a microsphere results in little toxic effects to the host cell, tissue, and the patient (in the case of *in vivo* use).

Intracellular delivery and transfection in accordance with the methods of the present invention may be performed *in vivo*, *ex vivo*, and *in vitro*. Included within the above methods is human gene therapy including wherein cells to be treated are excised from a patient. The cells are treated with an appropriate nucleic acid sequence and transfection with ultrasound is carried out in cell culture. The transfected cells are analyzed for increased synthesis of the appropriate nucleic acid sequence. The successfully transfected cells, measured by increases nucleic acid synthesis are then returned to the body of the patient. Transfection with ultrasound thereby results in the treatment of diseases by gene therapy. Diseases to be treated with the methods of the present invention include and are not limited to acquired immune deficiency syndrome, autoimmune diseases, chronic viral infection, hemophilia, muscular dystrophy, cystic fibrosis, diabetes, atherosclerosis, and cancer including and not limited to liver cancer, lung cancer, prostate cancer, ovarian cancer, brain cancer, kidney cancer, melanoma, neuroblastoma, and breast cancer. Many other diseases may, of course, be treated with the methods of the present invention, as will be apparent to the skilled artisan upon reading the present disclosure, and the treatment of all such diseases are to be considered within the scope of the present methods.

The use of heat, for example in the form of ultrasound, lithotripsy shock waves, and increased body temperature, in the present invention is useful in aiding the

delivery of compounds, such as, for example, nucleic acid sequences, into cells for therapeutic purposes. The introduction of a nucleic acid sequence into the cell may be the first step in incorporating the sequence into the genome. Such transfection techniques may be useful in conjunction with testing the range of ultrasound frequency useful in inducing the delivery of nucleic acid sequences to cells.

A method of identifying a nucleic acid sequence exhibiting increased synthesis comprising administering to a cell ultrasound for a time sufficient to increase synthesis of the nucleic acid sequence and observing an increase in synthesis of the nucleic acid sequence is embodied by the present invention.

Methods of treating a condition in a human subject comprising administering ultrasound to the subject such that synthesis of a nucleic acid sequence in the subject is increased, thereby treating the subject, are also contemplated by the present invention. In such methods, an portable ultrasound device, such as one worn by the subject, may deliver ultrasound at appropriate times without inconvenience to the subject. The ultrasound device may be programmed for dosing with ultrasound at specified intervals such that the device is automatically activated at predetermined intervals. Alternatively, the ultrasound device may be manually activated by the subject such that the subject controls the administration of ultrasound.

A method of treating a subject suspected of having phenylketonuria comprising administering to said human subject an exogenous nucleic acid sequence encoding phenylalanine hydroxylase and a therapeutically effective amount of ultrasound for a therapeutically effective amount of time such that ultrasound increases synthesis of phenylalanine hydroxylase nucleic acid sequence is also embodied by the present invention. Treating phenylketonuria may preferably include an exogenous nucleic acid sequence administered in a solid porous matrix of 1:3 DPEPC: dioleoylphosphatidylethanolamine (DOPE) and perfluorohexane, wherein the matrix comprises a plasmid having a phenylalanine hydroxylase nucleic acid sequence.

Also contemplated by the present invention is a method for increasing synthesis of a nucleic acid sequence encoding tumor suppressor gene p53 in a human subject comprising administering to the human subject an exogenous nucleic acid sequence encoding p53 and a therapeutically effective amount of ultrasound for a therapeutically effective

amount of time such that ultrasound increases synthesis of p53 nucleic acid sequence. The exogenous nucleic acid sequence is preferably administered in a solid porous matrix of 1:3 DPEPC: dioleoylphosphatidylethanolamine (DOPE) and perfluorohexane, wherein the matrix comprises a plasmid encoding p53.

Another embodiment of the present invention is a method for increasing synthesis of a nucleic acid sequence encoding IL-2 in a human subject comprising administering to the human subject an exogenous nucleic acid sequence encoding IL-2 and a therapeutically effective amount of ultrasound for a therapeutically effective amount of time such that ultrasound increases synthesis of an IL-2 nucleic acid sequence. IL-2 may associated with killer T lymphocytes. The exogenous nucleic acid sequence is preferably administered in a solid porous matrix of DMRIE/DOPE, wherein the matrix comprises a plasmid encoding IL-2.

A method of treating cancer is also embodied by the present invention and comprises administering to a human subject an exogenous antisense sequence of IF3, tRNA synthetase or a combination thereof, and a therapeutically effective amount of ultrasound for a therapeutically effective amount of time such that ultrasound increases synthesis of said antisense sequence. While not intending to be bound by any theory or theories of operation, it is believed that the antisense sequence for IF3 or tRNA synthetase will competitively interfere with protein synthesis and result in cell death.

A method of effecting a change in the expression of an endogenous nucleotide sequence in a cell comprising administering to said cell a therapeutically effective amount of ultrasound for a therapeutically effective time such that said administration of said ultrasound results in said change in expression of said nucleic acid sequence, wherein said endogenous nucleic acid sequence encoding a protein selected from the group consisting of a stress protein and a cellular repair protein is another embodiment of the present invention. A method of effecting the expression of an exogenous nucleotide sequence in a cell comprising administering to the cell a nucleic acid sequence and applying a therapeutically effective amount of ultrasound for a therapeutically effective time such that said administration of said ultrasound results in expression of said nucleic acid sequence is also contemplated by the present invention.

The methods of the present invention include increasing nucleic acid synthesis of all or part of a sense or an antisense sequence for the sequences identified herein including initiation factor 3 (IF3), tRNA synthetases, insulin (Giddings and Carnaghi, *Mol. Endocrinol.* **1990** *4*:1363-1369), Bcl 2 (Tsujimoto, Y., *et al.*, *PNAS, USA* **1986**, *83*:5214-5218), human leukocyte antigen (Trucco, G., *et al.*, *Diabetes* **1989**, *38*:1617-1622, thymidine kinase (Axel, R., et al., *J. Supramol. Struct.* **1979**, *8* (Suppl. 3):41), HLA-B7, Factor VIII (Higuchi, M., *et al.*, *Genomics* **1990**, *6*:65-71, ras/p53 (Arai, N., *et al.*, *Mol Cell Biol* **1986**, *6*:3232-3239, Mitsudomi, T., *et al.*, *Chest* **1993**, *104*:362-365), high density lipoprotein (hdl), leutinizing hormone releasing hormone (Maier, C.C., *et al.*, *Cell Mol Neurobiol* **1992**, *12*:447-454) and leutinizing hormone releasing hormone antagonist, antitumoral agents such as and not limited to insulin-like growth factor-1 (IGF-1, Barnes, M., *et al.*, *Obstetrics and Gynecology* **1997**, *89*:145-155), anti-IGF-1 (human IGF-1 gene fragment from published patent application GB2241703 GenBank accession number A29119), anti-k-ras (dog spleen mRNA 212 nucleotides GenBank accession number S42999), anti-c-fos (Rattus norvegicus Sprague Dawley c-fos gene, 5' flanking region GenBank accession number U02631), bcr-abl (Barnes, M., *et al.*, *Obstetrics and Gynecology* **1997**, *89*:145-155), c-myc (mouse c-myc gene, exons 1 and 2 GenBank accession number L00038, J00373, and J00374), c-myc promoter (Barnes, M., *et al.*, *Obstetrics and Gynecology* **1997**, *89*:145-155), erbB-2 promoter (Barnes, M., *et al.*, *Obstetrics and Gynecology* **1997**, *89*:145-155), erbB2 promoter-cytosine deaminase (human c-erb B2/neu protein gene, partial cDNA (CDs) GenBank accession number M95667), and antivirals such as and not limited to anti-human papilloma virus (HPV), anti-human immunodeficiency virus (HIV) such as HIVenv+rev (HIV type 1, isolate BTSPR, env gene, C2V3 region, partial CDs GenBank accession number U53195), tar/Td-rev (HIV type 1 rev-1 gene, 5' end GenBank accession number M38031, synthetic HIV1 TAR, 5' end GenBank accession number M27943), ribozyme, zeta-chimpanzee receptor, and the like, and all or part of a sequence encoding cytokines such as and not limited to interleukin 2 (IL-2) (human brain MRNA 418 nucleotides GenBank accession number S77835), interleukin 4 (Arai, N., *et al.*, *J Immunol* **1989**, *142*:274-282), interleukin 7 (human gene, exon 1 GenBank accession number M29048), interleukin 12 (mouse 5' flanking region of IL-12 p35 gene GenBank accession number D63334), interleukin 4 (human IL-4 gene, complete CDs GenBank accession number M23442), interleukin 6 (human gene for nuclear factor NF-IL-6

GenBank accession number X52560); gp130 (LIF receptor/IL-6 receptor complex component MRNA 150 nucleotides GenBank accession number S80479), interleukin 6 receptor, granulocyte macrophage colony stimulating factor (GM-CSF) (human GM-CSF gene, 5' flanking/promoter region GenBank accession number U31279), interferon including interferon gamma (human immune IFN-γ gene, complete CDs GenBank accession number J00219, M37265, V00536), tumor necrosis factor beta, TNF-β, (human 5' sequence of TNF-β gene GenBank accession number X59351)), vascular endothelial growth factor (VEGF), human growth hormone (hGH, Fidders, J.C., *et al., Proc Natl Acad Sci (USA)* **1979** *76*:4294-4298), colony stimulating factor, Factor VIII, Factor IX, Factor X, and the like. Other sequences useful in the methods of the present invention include ribozymes including catalytic RNA which may have a hammerhead secondary structure (Bratty, *et al., Biochim. Biophys. Acta* **1993** *1216*:345-349 and McKay, D.B., *RNA* **1996** *2*:395-403), c-myc, c-myb, tumor suppressor genes such as and not limited to human tumor antigen p53 (5' end GenBank accession number M26864), genes offering chemoprotection such as and not limited to those encoding multidrug resistance protein (MDR) (human mdr1 gene GenBank accession number X78081), genes for antigen overexpression such as and not limited to HLA-B7 (beta 2 microglobulin) (mouse MHC class I HLA-B7 gene, 5' flanking region GenBank accession M35971), carcinoembryonic antigen (CEA) (human 5' region GenBank accession number U17131), suicide genes such as and not limited to thymidine kinase (TK) (human TK gene encoding TK and promoter region GenBank accession number M13643), Ras, gene complementation genes such as and not limited to cystic fibrosis transmembrane conductance regulator (CFTR) (human CFTR gene, exon 1 GenBank accession number M55106 and M55499), adenosine deaminase (ADA) (human ADA gene, complete CDs GenBank accession number M13792), glucocerebrosidase, IRAP/TK (human MRNA for IRAP GenBank accession number X53296), vascular endothelial growth factor (VEGF) (mus musculus VEGF gene, partial CDs and promoter region GenBank accession number U41383), LDLR (human LDL receptor gene fragment GenBank accession number M60949), Fanconi Anemia Complementation Group C (FACC) (human FACC gene, 5' region GenBank accession number X83116), p47-phox (human P47 LBC oncogene MRNA, complete CDs GenBank accession number U03634), Factor IX (human Factor IX gene, exon 1 GenBank accession number K02048), α-1 antitrypsin (human α-1 antitrypsin gene S variant, complete CDs

GenBank accession number K02212), α-1 iduronidase (human iduronidase gene sequence GenBank accession number M88001), and iduronate sulfatase (Ids) (Mus musculus Ids MRNA, complete CDs GenBank accession number L07921), and gene markers such as and not limited to NeoR and LacZ, (bacteriophage T4 td gene, exon 2, 3' end; ORF2, complete CDs and ORF3, 5' end GenBank accession number M22627, and cloning vector pZEO (isolate SVLacZ) β-galactosidase (lacZ) gene, phleomycin/zeocin-binding protein (ShBle) gene, (complete CDs GenBank accession number L36850).

DNA encoding certain proteins may be used in the treatment of many different types of diseases. For example, adenosine deaminase may be provided to treat ADA deficiency; tumor necrosis factor and/or interleukin-2 may be provided to treat cancers; HDL receptor may be provided to treat liver disease; thymidine kinase may be provided to treat ovarian cancer, brain tumors, or HIV infection; HLA-B7 may be provided to treat malignant melanoma; interleukin-2 may be provided to treat neuroblastoma, malignant melanoma, or kidney cancer; interleukin-4 may be provided to treat cancer; HIV env may be provided to treat HIV infection; antisense ras/p53 may be provided to treat lung cancer; and Factor VIII may be provided to treat Hemophilia B. See, for example, Thompson, L., *Science*, **1992**, *258*, 744-746. Nucleotide sequences for the above-identified proteins are available in the scientific literature, including GENBANK, and are known to skilled artisans.

In addition to a coding sequence or antisense sequence, the nucleotide sequence administered to cells may have additional sequences to assist in the expression of the sequence. Suitable expression vectors, promoters, enhancers, and other expression control elements are known in the art and may be found in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, second edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989). Promoters such as and not limited to SV40, RSV, CMV, cd5k, IL5R α pgk-1, srα, TK, and the like are useful in the present invention. Transcription and/or translation control elements may be operatively linked to the sequence. For example, in an upstream position, a promoter may be followed by a translation initiation signal, comprising a ribosome binding site and an initiation codon, and in a downstream position may be a transcription termination signal. The transcription and translation control elements may be ligated in any functional combination or order. The transcription and translation control elements used in any particular embodiment of the invention will be chosen with reference

to the type of cell into which the expression vector will be introduced, so that an expression system is created. The selection of promoters, enhancers, and other expression control elements and the preparation of expression vectors suitable for use in the present invention will be well within the ambit of one skilled in the art once armed with the present disclosure. Also, introduction of the expression vector incorporating a sequence into a host cell can be performed in a variety of ways known in the art.

Mammalian cells may be primed to be more susceptible to uptake of DNA for gene therapy by the addition of various media, buffers, and chemicals known to those of skill in the art and set forth in Sambrook, *supra*. Administration of nucleic acid sequences *in vivo* may include, if desired, more than one sequence. For example, a single carrier may contain more than one sequence or carriers containing different sequences may be co-administered. In addition, one sequence may be delivered in a carrier and another naked sequence coadministered. Additional sequences, such as promoter sequences, may be delivered together with a sequence for therapeutic delivery, to increase expression thereof. For example, a heat shock protein nucleic acid sequence is an example of a sequence that exhibits increased synthesis. The heat shock protein nucleic acid sequence may be used to increase expression of a second gene sequence, such as, for example, by fusing the sequences for the heat shock protein and the second gene together whereby they are under control of the same promoter.

A wide variety of compounds may also be delivered to cells in accordance with the methods of the invention. Such other compounds include various other bioactive agents. As used herein, "bioactive agent" refers to any substance which may be used in connection with an application that is therapeutic or diagnostic in nature, such as, for example, in methods for diagnosing the presence or absence of a disease in a patient or in methods for the treatment of disease in a patient. As used herein, "bioactive agent" refers also to substances which are capable of exerting a biological effect *in vitro*, *in vivo*, and/or *ex vivo*. The bioactive agents may be neutral, or positively or negatively charged, etc., as desired. Examples of suitable bioactive agents include diagnostic and pharmaceutical agents, including drugs, synthetic organic molecules, proteins, peptides, vitamins, steroids, steroid analogs; and also include genetic material, including nucleosides, nucleotides and polynucleotides.

The phrase "diagnostic agent", as used herein, refers to any agent which may be used in connection with methods for imaging an internal region of a patient and/or diagnosing the presence or absence of a disease in a patient. Exemplary diagnostic agents include, for example, contrast agents for use in connection with ultrasound imaging, magnetic resonance imaging or computed tomography imaging of a patient. Diagnostic agents may also include any other agents useful in facilitating diagnosis of a disease or other condition in a patient, whether or not imaging methodology is employed.

The terms "pharmaceutical agent" or "drug", as employed herein, refer to any therapeutic or prophylactic agent which may be used in the treatment (including the prevention, diagnosis, alleviation, or cure) of a malady, affliction, disease or injury in a patient. Therapeutically useful peptides, polypeptides and polynucleotides may be included within the meaning of the term pharmaceutical or drug, as are various other therapeutically useful organic or inorganic compounds.

Particular examples of pharmaceutical agents which may be delivered by the methods of the present invention include, but are not limited to: mitotic inhibitors such as the vinca alkaloids, radiopharmaceuticals such as radioactive iodine, phosphorus and cobalt isotopes; hormones such as progestins, estrogens and antiestrogens; anti-helminthics, antimalarials and antituberculosis drugs; biologicals such as immune sera, antitoxins and antivenins; rabies prophylaxis products; bacterial vaccines; viral vaccines; aminoglycosides; respiratory products such as xanthine derivatives, theophylline and aminophylline; thyroid therapeutics such as iodine salts and anti-thyroid agents; cardiovascular products including chelating agents and mercurial diuretics and cardiac glycosides; glucagon; blood products such as parenteral iron, hemin, hematoporphyrins and their derivatives; targeting ligands such as peptides, antibodies, and antibody fragments; biological response modifiers such as muramyl dipeptide, muramyl tripeptide, microbial cell wall components, lymphokines (e.g. bacterial endotoxin such as lipopolysaccharide and macrophage activation factor); subunits of bacteria (such as Mycobacteria and Cornebacteria); the synthetic dipeptide N-acetyl-muramyl-L-alanyl-D-isoglutamine; antifungal agents such as ketoconazole, nystatin, griseofulvin, flucytosine (5-fc), miconazole, and amphotericin B; toxins such as ricin; immunosuppressants such as cyclosporins; and antibiotics such as β-lactam and sulfazecin; hormones such as growth hormone, melanocyte stimulating hormone, estradiol,

beclomethasone dipropionate, betamethasone, betamethasone acetate, betamethasone sodium phosphate, betamethasone disodium phosphate, betamethasone sodium phosphate, cortisone acetate, dexamethasone, dexamethasone acetate, dexamethasone sodium phosphate, flunisolide, hydrocortisone, hydrocortisone acetate, hydrocortisone cypionate, hydrocortisone sodium phosphate, hydrocortisone sodium succinate, methylprednisolone, methylprednisolone acetate, methylprednisolone sodium succinate, paramethasone acetate, prednisolone, prednisolone acetate, prednisolone sodium phosphate, prednisolone tebutate, prednisone, triamcinolone, triamcinolone acetonide, triamcinolone diacetate, triamcinolone hexacetonide, fludrocortisone acetate, oxytocin, and vasopressin, as well as their derivatives; vitamins such as cyanocobalamin neionic acid; retinoids and derivatives such as retinol palmitate and α-tocopherol; peptides and enzymes such as manganese superoxide dismutase and alkaline phosphatase; anti-allergens such as amelexanox; anti-coagulation agents such as phenprocoumon and heparin; tissue plasminogen activators (TPA), streptokinase, and urokinase; circulatory drugs such as propranolol; metabolic potentiators such as glutathione; antibiotics such as p-aminosalicyclic acid, isoniazid, capreomycin sulfate cycloserine, ethambutol hydrochloride ethionamide, pyrazinamide, rifampin, streptomycin sulfate dapsone, chloramphenicol, neomycin, ceflacor, cefadroxil, cephalexin, cephadrine erythromycin, clindamycin, lincomycin, amoxicillin, ampicillin, bacampicillin, carbenicillin, dicloxicillin, cyclacillin, picloxicillin, hetacillin, methicillin, nafcililn, oxacillin, penicillin (G and V), ticarcillin rifampin and tetracycline; antivirals such as acyclovir, DDI, Foscarnet, zidovudine, ribavirin and vidarabine monohydrate; antianginals such as diliazem, nifedipine, verapamil, erythritol tetranitrate, isosorbide dinitrate, nitroglycerin (glyceryl trinitrate) and pentaerythritol tetranitrate; antiinflammatories such as difluisal, ibuprofen, indomethacin, meclofenamate, mefenamic acid, naproxen, oxyphenbutazone, phenylbutazone, piroxicam, sulindac, tolmetin, aspirin, and salicylates; antiprotozoans such as chloraquine, hydroxychloraquine, metranidazole, quinine and meglumine antimonate; antirheumatics such as penicillamine; narcotics such as paregoric; opiates such as codeine, heroin, methadone, morphine, and opium; cardiac glycosides such as deslanoside, digitoxin, digoxin, digitalin, and digitalis; neuromuscular blockers such as atracurium nesylate, gallamine triethiodide, hexaflorenium bromide, metrocurine iodide, pancurium bromide, succinylcholine chloride (suxamethonium chloride), tubocurarine chloride and vecuronium bromide; sedatives such

as amorbarital, amobarbital sodium, aprobarbital, butabarbital sodium, chloral hydrate, ethchlorvynol, ethinamate, flurazepam hydrochloride, glutethimide, methotrimeprazine hydrochloride, methyprylon, midazolam hydrochloride, paraldehyde, pentobarbital, pentobarbital sodium, secobarbital sodium, tulbutal, temazepam and trizolam; local anesthetics such as bupivacaine hydrochloride, chloroprocaine hydrochloride, etidocaine hydrochloride, lidocaine hydrochloride, mepivacaine hydrochloride, procaine hydrochloride, and tetracaine hydrochloride; general anaesthetics such as droperidol, etamine hydrochloride, methohexital sodium and thiopental sodium; antineoplastic agents such as methotrexate, fluorouracil, adriamycin, mitomycin, ansamitomycin, bleomycin, cystein arabinoside, arabinosyl adenine, mercaptopolylysine, vincristine, busulfan, chlorambucil, azidothymidine, melphalan (e.g. PAM, L-PAM or phenylalanine mustard), mercaptopurine, mitotane, procarbazine hydrochloride dactinomycin (actinomycin D), danorubicin hydrochloride, dosorubicin hydrochloride, taxol, plicamycin (mithramycin), aminoglutethimide, estramustine phosphate sodium, flutamide, leuprolide acetate, leuprolide acetate, megestrol acetate, tamoxifen citrate, testolactone, trilostane, amsacrine (m-AMSA), asparaginase, etoposide (VP-16), interferon α-2a, interferon α-2b, teniposide (VM-26), vinblastine sulfate (VLB), vincristine sulfate, hydroxyurea, procarbaxine, and dacarbazine.

Although a wide variety of compounds, including nucleotides, may be delivered in accordance with the present invention, preferably the nucleotides are less than about 10,000 bases (or base pairs) in length, more preferably between about 20 to about 10,000 bases (or base pairs) in length, even more preferably between about 2,000 and about 8,000 bases (or base pairs) in length, and most preferably between about 4,000 and 6,000 bases (or base pairs) in length. Other (non-nucleotide) compounds or bioactive agents to be delivered are preferably less than about 5000 kilodaltons (5000 kD) in molecular weight, more preferably between about 10 and about 1000 kD, even more preferably between about 100 and about 500 kD. As one skilled in the art will recognize, however, larger and smaller sized compounds may also be delivered in accordance with the present invention.

The useful dosage of nucleic acid sequences or other compounds to be administered or delivered, as well as the mode of administration, will vary depending upon type and nature of the compound to be delivered, the age, weight, cells or patient (animal) to be treated, the particular diagnostic, therapeutic, or other application intended (including the

disease state, if any, to be treated), and the organic halide (if any) and carrier (if any) employed. Typically, dosage is initiated at lower levels and may be increased until the desired therapeutic effect is achieved. The desired dosage, including any therapeutically or diagnostically effective dosage amounts, will be well within the ambit of one skilled in the art, armed with the prevailing medical literature and with the present disclosure. Representative amounts are provided in the examples herein. Of course, higher or lower amounts may be employed, as will be recognized by the skilled artisan.

As one skilled in the art would recognize, administration of compositions of the present invention may be carried out in various fashions, such as intravascularly, intralymphatically, parenterally, subcutaneously, intramuscularly, intranasally, intrarectally, intraperitoneally, interstitially, into the airways via nebulizer, hyperbarically, orally, topically, or intratumorly, using a variety of dosage forms. One method of topical administration is the addition of a nucleic acid sequence (or other compound to be delivered), preferably in a carrier such as and not limited to a hydrogel, applied to the outside of a balloon catheter. The catheter is inserted into the blood stream of a patient. Once the balloon of the catheter reaches the location to which the sequence is to be administered, the balloon is pumped up and the sequence-containing hydrogel adheres to the blood vessel surface thus delivering the sequence. In addition, ultrasound may be applied to the cells endoscopically and intravascularly, for example, as well as, of course, applied externally.

A number of transfection and other intracellular delivery techniques are possible in accordance with the methods of the present invention employing the subject methods and the organic halides and/or carriers as disclosed herein. Two methods, using calcium phosphate and viral vectors, are indirect methods of introducing the nucleotide sequence into cells because they involve the passive uptake of the nucleotide sequence by the cell which is to be transfected.

Calcium phosphate coprecipitation is a chemical-mediated indirect method of transfection. The nucleic acid sequence (or other compound to be administered) is introduced into mammalian cells, for example, by coprecipitation of the sequence with calcium phosphate, calcium chloride, calcium hydroxybutarate, and the like; then the mixture is presented to the cells. The purified nucleic acid sequence is mixed with buffers containing phosphate and calcium chloride which results in the formation of a very fine precipitate, and

the mixture is presented to the cells in culture. A protocol for cells that grow attached to a substratum as set forth in Keown, W. A., *et al.*, "Methods for Introducing DNA into Mammalian Cells," in *Methods in Enzymology, Vol. 185, Gene Expression Technology*, Ed., Goeddel, David V., pp. 527-537, Academic Press, Inc., New York, New York, 1991 is incorporated herein by reference in its entirety. Briefly, on day 1, cells are seeded at 2-3 X 10^4 cells/cm^2 in normal growth medium and allowed to attach. At the time of transfecting, the cells should be 80-90% confluent. On day 2, the nucleic acid sequence-calcium phosphate coprecipitate is prepared, mixed and allowed to stand at room temperature for about 30 minutes. The nucleic acid sequence is added to TE buffer (10 mM tris, 1 mM EDTA, pH 8.0), 2X HBAS (Hanks' balanced slats, 1.4 mM Na_2HPO_4, 10 mM KCl, 12 mM glucose, 275 mM NaCl, and 40 mM HEPES, pH 6.95), and 2M $CaCl_2$ (calcium chloride in 10 mM HEPES, pH 5.8). The medium is removed from the cells and replaced with fresh medium. The precipitate is mixed gently by shaking or pipetting and added directly to the medium in dishes containing cells. The cells are incubated at 37° C for 4 hours. The medium containing the precipitate is removed and dimethyl sulfoxide in 1 X HBS. After 2 minutes, 4 ml of serum-free medium is added to each dish. The mixture is aspirated, washed twice with serum-free medium, and medium is added and incubated overnight at 37° C. The cells are trypsinized and the contents of each plate is split into 3-4 new plates. Selection may be applied for stable transfectants, in which selective medium may be used at this time or a day later.

The present invention employing the methods of the invention and the organic halides and/or carriers may also be useful concurrently with microinjection and electroporation. Microinjection involves the direct microinjection of nucleic acid sequences into the nucleus of a host cell. Microinjection does not expose the nucleic acid sequence to the cytoplasm or organelles within it. This is beneficial since considerable damage may result to the DNA during transit from the cell exterior to the nucleus. Electroporation involves electric field-mediated nucleic acid sequence transfection. When membranes are subjected to an electric field of sufficiently high voltage, regions of the membrane undergo a reversible breakdown, resulting in the formation of pores large enough to permit the passage of nucleotide sequences. Electroporated nucleic acid sequences remain free in the cytosol and nucleoplasm. Very few copies of transfected nucleic acid sequences may be introduced with

electroporation. Cells susceptible to electroporation include, for example, lymphocytes, hematopoietic stem cells, and rat hepatoma cells.

In accordance with the present invention, for *in vivo* applications, a lower frequency of ultrasound is usually selected for cells of deep seated or thick tissues, e.g. transcutaneous application of ultrasound to cells of the deep seated muscle or organs in the abdomen or retroperitoneum. For cells of small tissues a higher frequency of sound energy is applied, e.g. for the eye. For intravascular applications, which may employ intravascular catheters equipped with ultrasound transducers for endovascular gene therapy, higher frequencies may be employed such as over about 20 megahertz. For most applications however the frequency of the sound ranges from about 500 kilohertz to about 3 megahertz, preferably from about 500 kilohertz to about 1 megahertz, more preferably about 200 kilohertz, more preferably about 40 kilohertz to about 25 megahertz, even more preferably about 10 megahertz. Compared to lithotripsy, the frequency employed in the present invention is more than about 2 or 3 orders of magnitude higher and the energy levels of the present invention are lower.

The sound energy is applied in waves of sonic energy over a given duty cycle (sometimes referred to as pulse duration) and level of intensity. Generally continuous wave ultrasound which applies a constant train of ultrasound pulses is employed. The duty cycle is selected so that the level of energy output is in a desired range. The duty cycle may be varied from between 1% and 100% meaning that the ultrasound energy will be pulsing from between 1% and 100% of the time. For example, a period of ultrasound treatment may take place over 25 minutes with three duty cycles of ultrasound, each five minutes in duration, interrupted by two periods of no ultrasound. Preferably the duty cycle is 100%, more preferably about 75%, more preferably about 50%, even more preferably about 20%, even more preferably about 15%, and even more preferably about 10%.

Ultrasound for use in the present invention is typically provided at a frequency lower than the frequency used for imaging by ultrasound. The frequency of ultrasound which is selected will vary depending upon the location of cells which are being transfected, and or other factors that will be readily apparent to one skilled in the art based upon the present disclosure. In addition to frequency, the energy level (sometimes referred to as power intensity or power level) also has a large effect on total energy which is applied to the cells

or tissue for ultrasound enhanced transfection. Suitable energy levels will be readily apparent to one skilled in the art based upon the present disclosure. Typically, the energy level settings are somewhat higher than employed in diagnostic ultrasound but may range from about 500 milliwatts/cm^2 to about 10 watts/cm^2, more preferably from about 200 milliwatts/cm^2 to about 10 milliwatts/cm^2, and more preferably of from about 50 milliwatts/cm^2 to about 2 watts/cm^2. The power level which is applied is selected so that both peak spatial temporal power and total energy deposition is generally below the cytotoxic threshold for the cells or tissue. Generally, frequencies and energy levels are applied at lower amounts, then increased until the desired cellular uptake of the administered compound is achieved.

As one skilled in the art would recognize, high energies of ultrasound may be used for hyperthermia to heat the tissue and also to directly ablate tissues with very high levels of energy. In the ultrasound enhanced transfection and gene expression of the present invention, energy levels are far below those which cause tissue ablation and below those which cause a significant hyperthermic effect. As one skilled in the art would recognize once armed with the present disclosure, energy deposition is a function of both power intensity and duty cycle. Higher spatial peak temporal average power tends to shift the bioeffect curve such that lower total energy may be applied to create a greater bioeffect. Higher energy levels and lower ultrasonic frequencies are required for penetration into deep seated tissues; conversely lower energy levels and higher ultrasonic frequencies are needed for treatment of superficial tissues or when the ultrasound transducer can be applied directly to the tissue surface. Small volume cell culture samples need less power for ultrasound enhanced transfection than large volume bioreactor chambers which may be multiple liters in size and therefore need higher energy levels to enhance gene expression. The geometry of a cell culture container will also affect the ultrasound energy requirements.

In accordance with the present invention, ultrasound energy may be used to increase the synthesis of a nucleic acid sequence. In addition, inducing a cell to take up a nucleic acid sequence is enhanced by the application of ultrasound.

The ultrasound energy may be applied to the tissue or cells either before, simultaneously with, or after administration of the compound to the cell, preferably simultaneously with or after. Typically the ultrasound energy is applied from no more than about 48 hours prior to administration of the nucleic acid sequence to the cells and/or up to

no more than about 48 hours after the sequence has been administered to the cell, although longer or shorter times may be applied. More preferably, the ultrasound energy is applied at some time or at various time points from about 4 hours before administration of the compound or genetic material up to about 24 hours after administration. Most preferably the ultrasound energy is applied within about 1 hour prior to transfection up to about 12 hours post transfection.

Either one or multiple applications of ultrasound energy may be employed. The duration of ultrasound energy exposure (exposure time) will vary depending upon the power level of the ultrasound and the duty cycle. To determine the preferable duration, ultrasound is typically applied at lower exposure times, and increased until the desired cellular uptake of the compound administered is achieved. A high intensity (high power level; typically greater than about 2 watts/cm^2, preferably over about 5 watts/cm^2, and also preferably over about 10 watts/cm^2, depending on the pulse duration) ultrasound shock wave may require only a few milliseconds of exposure. This may also be the case when cavitation nuclei such as gas filled liposomes or perfluorocarbon emulsions are present within the medium. A very brief exposure to high energy ultrasound may be sufficient to enhance transfection. The presence of cavitation nuclei in the transfection medium will lower the cavitation threshold and therefore potentially decrease energy requirements for ultrasound enhanced transfection as well as to potentially decrease the necessary exposure time. More typically the exposure time ranges from about a few seconds to up to about an hour of ultrasound energy application to the cell to achieve most effective ultrasound enhanced gene transfection. Even more preferably the duration of ultrasound exposure ranges from about a few seconds to about a few minutes and may be repeated at various intervals during transfection. The duration of ultrasound energy exposure should be sufficient to cause the desired effect but not so long that significant cytotoxicity may result.

The effect of ultrasound enhanced transfection is independent of hyperthermia. While the application of ultrasound energy necessary to increase the efficiency of transfection may result in a few degrees centigrade increase in temperature, any increase in temperature is typically transient and the temperature rapidly returns to baseline. More preferably the temperature does not increase significantly during application of the ultrasound. An increase in temperature is typically less than about 1° C to about 2° C. Progressively higher levels of

ultrasound energy will result in progressive rises in temperature but temperature is preferably maintained below the level where significant cytotoxicity will occur (e.g. 44° C or higher). As one may note, the sample measures the temperature in a solution of normal saline when exposed to ultrasound. The applied energy is 10 watts imparted through a 5.0 cm^2 transducer, or 2 W cm^{-2}. Sound energy from the ultrasound transducer may be simply converted to thermal energy in the aqueous milieu. The amount of energy and/or the time of exposure may be modified so as to prevent temperature-induced cell destruction.

The ultrasound energy may be applied with any of a variety of commercially available ultrasound systems. For example a Rich-Mar model 25 ultrasonic therapy apparatus (Rich-Mar Corporation, Inola, Oklahoma) with the center frequency residing at approximately 1.0 MHz, in pulsed or continuous mode, may be used to practice the invention. Conventionally available transducers, power amplifiers and other component systems for practicing the invention can also be readily assembled. Wave synthesizers and pulsers may also be incorporated into the system to allow control over the pulse repetition intervals, duty cycles, etc. Advantageously, these components can also be used to modify the ultrasound pulses to employ varying frequency and amplitude effects such as CHIRP (increasing in frequency) and PRICH pulses (decreasing in frequency) waveform patterns. Ultrasonic energies can also be supplied from commercially available amplifiers, transducers and frequency generators. By way of example, a power transducer with a center frequency of 1.0 MHz from Valpey-Fisher (Valpey-Fisher, Hopkinton, Mass.), a power RF amplifier from ENI (ENI, Rochester, New York), and a function generator from Hewlett Packard (Hewlett Packard, Sunnyvale, Calif.) may be a suitable setup to accomplish the above goals. Alternatively, a pulse/function generator or an arbitrary function generator may also be used to accomplish variable pulse formats. In addition, methods that would allow for gating the various signals together, could conceivably be accomplished.

The high energy ultrasound system may also be incorporated with ultrasound imaging such as described in U.S. patent application U.S. Serial No. 08/468,052, filed June 6, 1995, and the disclosures of which are hereby incorporated herein by reference in their entirety. Also application of high energy ultrasound may be performed under other forms of conventional imaging such as endoscopy (e.g. fiberoptic), computed tomography, magnetic resonance imaging, angiography, and nuclear medicine. Such imaging may be employed, for

example, to locate and identify in a patient the cells to which the ultrasound induced (or other) heating should be applied, or used to follow and/or locate the composition of the invention after administration to a patient. The ultrasound may be applied so as to effectively create second harmonic superimposition on the target treatment zone of tissue to increase the effectiveness of transfection. For example, a prototype sector-vortex phased array transducer, depicted in Figure 5, 120 mm in diameter, which generates 750 kHz and 1.5 MHZ ultrasound may be employed. As described in the reference by K. Kawabata and S. Umemura, *Ultrasonics Sonochemistry* **1996**, *3*:1-5, a transducer may be constructed with 32 piezoelectric (PZT) transducer elements from lead zirconate PZT material. The transducer may be constructed in two tracks such that there are 16 sectors in each track. The lower frequency ultrasound could be applied from the outer track and the higher frequency, 1.5 MHZ, from the inner track. A shell may be constructed with a 120 mm radius of curvature for geometric focusing. The beam profile provided by the piezoelectric elements and spherical shape of the transducer cell assembly can be designed so as to superimpose the focal zones of the two different frequencies of ultrasound. This may result in focal acoustic power with superimposition of the lower frequency and higher frequency ultrasound sources. This results effectively in second harmonic superimposition of the ultrasound signal. While not intending to be bound by any particular theory of operation, it is believed that this ultrasound assembly will allow for improved transfection efficiency at lower total amounts of energy and thus result in reducing damage to the cell.

Skilled artisans would recognize, once armed with the present disclosure, that the two ultrasound energy sources may be at other frequencies such that the first source (low frequency) is one half the frequency of the second source. For example, 500 kHz in the outer assembly and 1 MHZ in the inner assembly may be employed. A range of different frequencies may be selected such that the outer assembly is 1X and the inner assembly is 2X. Alternatively, the assemblies may be designed such that the higher frequency is in the outer assembly and the lower frequency is in the inner assembly. Alternatively still, odd harmonics may be utilized such that the outer and inner tracks may be represented by X and 3X frequencies or X and 5X frequencies. The second harmonic, ultraharmonic, or subharmonic frequencies are superimposed at the focal zone which is directed towards the target tissues or cells to be transfected. Thus, ultrasound may be administered simultaneously at two or more

frequencies to result in superimposition of ultrasonic frequencies, including and not limited to second harmonic frequencies.

The invention is further demonstrated in the following actual Examples 1 and 8 and prophetic Examples 2-7.

EXAMPLE 1

In order to delineate the effects of ultrasound on genetic expression at the molecular level, the following experiment was designed. The cell line used was NIH/3T3.

Thirty three (33) PCR primers, were designed to amplify genes involved in cell repair and hat shock response. The sequences were downloaded from the National Institute of Health's National Center for Biological Information GENBANK database. The sequences were loaded into a software package that analyzed for optimal PCR primers. (Oligo 5.0, National Biosciences, Plymouth, MN). Once primers were identified, they were matched to the database to make certain that they would only amplify the target sequence. Cells were exposed to 0.5 W/cm2 of ultrasound energy for 30 seconds (RichMar model 25, Enola, OK) and total RNA was extracted using Tripure reagent (Boehringer Mannheim Biochemicals, Indianapolis, IN (BMB)) at four (4) time points: One hour post ultrasound application, 4 hours post, 24 hours post, and 48 hours post application. The messenger RNA was extracted from the total RNA using a magnetic particle isolation system (BMB). Reverse transcriptase PCR was then carried out to determine which if any of the cell repair genes had been upregulated. In reverse transcriptase PCR, the mRNA is first converted into the complementary DNA and then amplified in the polymerase chain reaction. This was carried out using the Titan™One-Tube rt-PCR system (BMB) and a Stratagene RoboCycler PCR machine (LaJolla, CA). The samples were loaded onto a 1% agarose gel in an Owl Scientific A5 gel box (Woburn, MA) and run for 3 hours at 110 volts supplied by an International Biotechnologies, Inc. MBP300 power supply (New Haven, CT). The gel was then stained with ethidium bromide (Life Technologies, Gaithersburg, MD) and placed on an ultraviolet transilluminator (Ultra-Lum, Inc., Carson, CA). Photographs of the gel were taken with a Polaroid DS34 camera with EPH13 hood (Norwood, MA).

Results: NIH/3T3 Sequences Transcribed without Ultrasound as Amplified by PCR Primers: Ca+2 ATPase (SEQ ID NOS: 19 and 20), ERCC1 (SEQ ID NOS: 23 and

24), Heme Oxygenase (SEQ ID NOS: 35 and 36), Rad23 (HHR23B) (SEQ ID NOS: 45 and 46), Raf (SEQ ID NOS: 47 and 48), and TCP-1-B (SEQ ID NOS: 55 and 56); NIH/3T3 Sequences Transcribed with Ultrasound as Amplified by PCR Primers: c-fos (SEQ ID NOS: 1 and 2), calsequestrin (SEQ ID NOS: 7 and 8), β-polymerase (SEQ ID NOS: 13 and 14), 3-methyladenine DNA glycosylase (SEQ ID NOS: 15 and 16), Ca+2 ATPase (SEQ ID NOS: 19 and 20), ERCC1 (SEQ ID NOS: 23 and 24), heat shock protein 27 (SEQ ID NOS: 29 and 30), Heme Oxygenase (SEQ ID NOS: 35 and 36), heat shock protein 89α (SEQ ID NOS: 37 and 38), MAP-kinase kinase (SEQ ID NOS: 41 and 42), pericentrin (SEQ ID NOS: 43 and 44), Rad23 (HHR23B) (SEQ ID NOS: 45 and 46), Raf (SEQ ID NOS: 47 and 48), Ras (SEQ ID NOS: 51 and 52), TCP-1-B (SEQ ID NOS: 55 and 56), ubiquinone oxidoreductase complex (SEQ ID NOS: 57 and 58), ubiquitin (SEQ ID NOS: 59 and 60), XPA (SEQ ID NOS: 61 and 62), and XPB (SEQ ID NOS: 63 and 64). The genes which are amplified by the PCR primers constructed for each of the tested genes are visible in Figure 2.

EXAMPLE 2 - Therapy for Phenylketonuria

A tandem construct of a plasmid is developed with the ras gene upstream from the phenylalanine hydroxylase gene. The plasmid construct is complexed with 1:3 DPEPC:DOPE with DNA/lipid ratio of 1:2 v/v with a perfluorohexane core. Three ccs of a suspension of the material is injected into the muscle and liver of a patient with phenylketonuria per treatment dose. Local ultrasound is applied at 1.0 MHZ and 0.5 watts/cm2 for 60 seconds at varying time intervals to stimulate production of phenylalanine hydroxylase.

EXAMPLE 3 - Enhancing expression of tumor suppressor genes-based therapy

A tandem construct of hsp27 is prepared with RSV promoter with the hsp gene upstream from p53. This is complexed with 1:3 DPEPC:DOPE with DNA/lipid ratio of 1:2 v/v with a perfluorohexane core and administered IV at a DNA dose of 1500 micrograms and 0.5 MHZ continuous wave ultrasound is applied to liver metastases transcutaneously at 2 watts/cm2 with 10% duty cycle for five minutes following IV injection of the composition. p53 gene is expressed locally in the tumor cells and surrounding tissue causing regression of

the tumor and suppression of metastasis. Ultrasound ma be reapplied at daily intervals for the next two weeks to increase production of p53.

EXAMPLE 4 - Enhancing expression of IL2 on killer T cells

A tandem construct plasmid was prepared with CMV promoter and c-fos upstream of the IL-2 gene. The plasmid DNA is complexed with DMRIE/DOPE at a lipid/DNA ratio of 1:6. In a patient with malignant melanoma 1500 micrograms of plasmid DNA is injected into metastatic tumor in the retroperitoneum under CT guidance. Twenty-four hours later, ultrasound imaging is performed and focused high intensity ultrasound (1.5 MHZ, 2 watts/cm2) is applied to the tumor mass for 60 seconds. The result is increased intratumoral production of IL-2 and tumor mass reduction.

EXAMPLE 5

A patient is injected in the pectoralis major muscle with 1500 micrograms of gene in a plasmid containing a plasmid construct with an upstream HIV promoter and structural genes for c-fos and just downstream the gene for human growth hormone (hGH). Forty-eight hours after the gene is injected intramuscularly, the patient is fitted with a portable ultrasound transducer ensemble. The transducer ensemble comprises a 60kHz transducer which is attached to the patient's skin via a suction cup. The transducer is powered by a battery pack, miniature amplifier and pulse generator. Periodically, e.g., every 6 to 12 hours, 2 minutes of ultrasound is applied to the patient's chest wall such that insonation is applied to the tissue where transfection occurs. The result is pulsation production of growth hormone. The power output and pulsing intervals of the ultrasound application can be adjusted for maximal therapeutic effectiveness.

EXAMPLE 6

A patient with diabetes is injected in the thigh muscle with a plasmid which contains a promoter for the cell repair gene (e.g. *c-fos* or *c-jun*) upstream from the gene for proinsulin. The patient is fitted with a portable ultrasound ensemble equipped with a 1-megahertz transducer. The patient is also equipped with a glucometer (Elite Diabetes Care System, Miles Diagnostics, Inc., Tarrytown, NY). The glucometer is designed to send a

signal to the transducer ensemble, when blood glucose levels rise beyond a target level, e.g., 140 mg/dL. At glucose levels in excess of this threshold, the ultrasound apparatus is triggered to apply a pulse of sonic energy to the tissue which has been transfected with the ultrasound sensitive promoter and gene proinsulin. The result is tight control over the patient's blood glucose level through control of the time frame of production of insulin.

EXAMPLE 7

A patient with Alzheimer's disease received an intra-cortical injection of a gene construct with the gene for nerve growth factor (hNGF). The gene is delivered with Herpes Simplex Virus which encapsulates an HSV promoter as well as the heat shock protein (hsp) promoter and the gene for hNGF. A burr hole is created in the skull and a 2 megahertz transducer is introduced into the epidural space. The transducer ensemble is designed to apply ultrasound diurnally to achieve physiological release of hNGF.

EXAMPLE 8

Six genes, 3-methyladenine glycosylase, β-polymerase, c-myc, Egr, HHR6A, and HSP60 (SEQ ID NOS: 67-72) were upregulated with ultrasound after 24 hours post exposure. This example is carried out in accordance with the procedure set forth in Example 1 with NIH/3T3 cells exposed to ultrasound at 0.5 W/cm^2 for 30 seconds. The RNA observed in the cells was then analyzed for homology overlap of the sequences by a software program (Lazergene, DNAstar, Madison, WI). Consensus sequences were generated from the upstream regions of each of the six genes. The consensus sequence of each of the six genes (SEQ ID NO: 73) was compared to the sequence in the GenBank library by BLAST to determine which other genes have a high homology with the consensus region. The two genes having greater than 90% homology to the consensus sequences were IF-3 and tRNA synthetase. Thus, IF-3 and tRNA synthetase are expected to be stimulated in transcription by ultrasound.

The disclosures of each patent, patent application and publication cited or described in this document are hereby incorporated herein by reference, in their entirety.

Various modifications of the invention, in addition to those described herein, will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

Claims

WHAT IS CLAIMED IS:

1. A method of increasing nucleic acid synthesis in a cell comprising administering to the cell a therapeutically effective amount of ultrasound for a therapeutically effective time such that said administration of said ultrasound results in said increased nucleic acid synthesis, wherein said nucleic acid comprises an endogenous sequence encoding a protein selected from the group consisting of a stress protein and a repair protein.

2. A method of increasing nucleic acid synthesis in a cell comprising administering to the cell a nucleic acid sequence and a therapeutically effective amount of ultrasound for a therapeutically effective time such that said administration of said ultrasound results in said increased nucleic acid synthesis, wherein said nucleic acid comprises an exogenous sequence.

3. A method of claim 1 wherein said protein is selected from the group consisting of a initiation factor 3, tRNA synthetase, heat shock protein, ubiquinone oxidoreductase, XPA nucleotide excision repair gene, XPB nucleotide excision repair gene, XPG nucleotide excision repair gene, Ca+2 ATPase, ERCC1, Heme Oxygenase, Rad23, Raf, TCP-1-B, calsequestrin, β-polymerase, 3-methyladenine DNA glycosylase, MAP-kinase kinase, pericentrin, ubiquitin, B2, Cox3, erg-1, HHR6B, HHR6A, RPA, SRC, catalase, and creatine.

4. A method of claim 3 wherein said heat shock protein is selected from the group consisting of heat shock protein 27, heat shock protein 89α, and heat shock protein 60.

5. A method of claim 2 wherein said exogenous sequence is selected from the group consisting of a proto-oncogene, stress protein, repair protein, and structural protein.

6. A method of claim 2 wherein said exogenous sequence is selected from the group consisting of initiation factor 3, tRNA synthetase, heat shock protein, ubiquinone oxidoreductase, XPA nucleotide excision repair gene, XPB nucleotide excision repair gene, XPG nucleotide excision repair gene, Ca+2 ATPase, ERCC1, Heme Oxygenase, Rad23, Raf,

TCP-1-B, calsequestrin, β-polymerase, 3-methyladenine DNA glycosylase, MAP-kinase kinase, pericentrin, ubiquitin, B2, Cox3, erg-1, HHR6B, HHR6A, RPA, SRC, catalase, creatine, Ras, c-fos, c-myc, c-jun, and jun-b.

7. A method of claim 6 wherein heat shock protein is selected from the group consisting of heat shock protein 27, heat shock protein 89α, and heat shock protein 60.

8. A method of claim 2 wherein said exogenous sequence is provided simultaneously with said ultrasound, prior to said ultrasound, or after said ultrasound.

9. A method of claim 1 or 2 wherein said ultrasound is provided in a range of about 5 to about 40 kilohertz.

10. A method of claim 1 or 2 wherein said ultrasound is provided in a range of about 10 to about 20 kilohertz.

11. A method of claim 1 or 2 wherein said ultrasound is provided at about 25 kilohertz.

12. A method of claim 1 or 2 wherein said nucleic acid is selected from the group consisting of DNA and RNA.

13. A method of claim 1 or 2 wherein said cell is an animal cell or a plant cell.

14. A method of claim 13 wherein said animal cell is a mammalian cell.

15. A method of claim 14 wherein said animal cell is a human cell.

16. A method of claim 1 or 2 wherein said synthesis is measured by amplification of said nucleotide sequence.

17. A method of claim 1 or 2 wherein said expression measured by amplification of cDNA, wherein said amplification comprises reverse transcriptase-Polymerase Chain Reaction.

18. A method of claim 1 or 2 wherein said therapeutically effective amount of time is from about 5 seconds to about 120 seconds.

19. A method of claim 1 or 2 wherein said therapeutically effective amount of time is from about 30 seconds.

20. A method of claim 2 wherein said exogenous sequence is provided together with an organic halide.

21. A method of claim 20 wherein said composition further comprises a carrier.

22. A method of claim 20 wherein said organic halide is selected from the group consisting of a gaseous organic halide and a liquid organic halide.

23. A method of claim 22 wherein said organic halide is a gas.

24. A method of claim 22 wherein said organic halide is a liquid.

25. A method of claim 20 wherein said organic halide is a gaseous precursor.

26. A method of claim 20 wherein said organic halide is a fluorinated compound.

27. A method of claim 20 wherein said organic halide is a perfluorinated compound.

28. A method of claim 20 wherein said organic halide is a perfluorocarbon.

29. A method of claim 20 wherein said organic halide is a perfluoroether compound.

30. A method of claim 28 wherein said perfluorocarbon is a liquid.

31. A method of claim 30 wherein said liquid perfluorocarbon is a gaseous precursor.

32. A method of claim 28 wherein said perfluorocarbon is a gas.

33. A method of claim 20 wherein said organic halide is selected from the group consisting of 1-bromo-nonafluorobutane, perfluorooctyliodide, perfluoroocytlbromide, 1-chloro-1-fluoro-1-bromomethane, 1,1,1-trichloro-2,2,2-trifluoroethane, 1,2-dichloro-2,2-difluoroethane, 1,1-dichloro-1,2-difluoroethane, 1,2-dichloro-1,1,3-trifluoropropane, 1-bromoperfluorobutane, 1-bromo-2,4-difluorobenzene, 2-iodo-1,1,1-trifluoroethane, 5-bromovaleryl chloride, 1,3-dichlorotetrafluoroacetone, bromine pentafluoride, 1-bromo-1,1,2,3,3,3- hexafluoropropane, 2-chloro 1,1,1,4,4,4-hexafluoro-2-butene, 2-chloropentafluoro-1,3-butadiene, iodotrifluoroethylene, 1,1,2-trifluoro-2-chloroethane, 1,2-difluorochloroethane, 1,1-difluoro-2-chloroethane, 1,1-dichlorofluoroethane, heptafluoro-2-iodopropane. bromotrifluoroethane, chlorotrifluoromethane, dichlorodifluoromethane, dibromofluoromethane, chloropentafluoroethane, bromochlorodifluoromethane. dichloro-1.1.2.2-tetrafluoroethane. 1,1,1,3,3-pentafluoropentane, perfluorotributylamine, perfluorotripropylamine, 3-fluorobenzaldehyde, 2-fluoro-5-nitrotoluene, 3-fluorostyrene, 3,5-difluoroaniline, 2,2,2-trifluoroethylacrylate, 3-(trifluoromethoxy)-acetophenone, 1,1,2,2,3,3,4,4-octafluorobutane, 1,1,1,3,3-pentafluorobutane, 1-fluorobutane, 1,1,2,2,3,3,4,4-octafluorobutane, 1,1,1,3,3-pentafluorobutane, perfluoro-4 methylquinolizidine, perfluoro-N-methyl-decahydroquinone, perfluoro-N-methyl-decahydroisoquinone, perfluoro-N-cyclohexyl-pyrrolidine, tetradecaperfluoroheptane, dodecaperfluorocyclohexane, perfluoromethane, perfluoroethane, perfluoropropane, perfluorobutane, perfluoropentane, perfluorohexane, perfluoroheptane, perfluorooctane, perfluorononane, perfluorodecane, perfluorododecane, perfluoro-2-methyl-2-pentene, perfluorocyclohexane, perfluorodecalin, perfluorododecalin, perfluoropropylene, perfluorocyclobutane, perfluoro-2-butyne, perfluoro-2-butene, perfluorobuta-1,3-diene,

perfluorobutylethyl ether, bis(perfluoroisopropyl) ether, bis(perfluoropropyl) ether, perfluorotetrahydropyran, perfluoromethyl tetrahydrofuran, perfluoro t-butyl methyl ether, perfluoro isobutyl methyl ether, perfluoro n-butyl methyl ether, perfluoro isopropyl ethyl ether, perfluoro n-propyl ethyl ether, perfluoro cyclobutyl methyl ether, perfluoro cyclopropyl ethyl ether, perfluoro isopropyl methyl ether, perfluoro n-propyl methyl ether, perflouro diethyl ether, perfluoro cyclopropyl methyl ether, perfluoro methyl ethyl ether, perfluoro dimethyl ether, sulfur hexafluoride, and selenium hexafluoride.

34. A method of claim 20 wherein said organic halide is selected from the group consisting of perfluoromethane, perfluoroethane, perfluoropropane, perfluorobutane, perfluoropentane, perfluorohexane, perfluoroheptane, perfluorooctane, perfluorononane, perfluorodecane, perfluorododecane, perfluoro-2-methyl-2-pentene, perfluorocyclohexane, perfluorodecalin, perfluorododecalin, tetradecaperfluorohexane, and dodecaperfluorocyclohexane.

35. A method of claim 20 wherein said organic halide is selected from the group consisting of perfluorobutylethyl ether, bis(perfluoroisopropyl) ether, bis(perfluoropropyl) ether, perfluorotetrahydropyran, perfluoromethyl tetrahydrofuran, perfluoro t-butyl methyl ether, perfluoro isobutyl methyl ether, perfluoro n-butyl methyl ether, perfluoro isopropyl ethyl ether, perfluoro n-propyl ethyl ether, perfluoro cyclobutyl methyl ether, perfluoro cyclopropyl ethyl ether, perfluoro isopropyl methyl ether, perfluoro n-propyl methyl ether, perflouro diethyl ether, perfluoro cyclopropyl methyl ether, perfluoro methyl ethyl ether, and perfluoro dimethyl ether.

36. A method of claim 20 wherein said organic halide is selected from the group consisting of perfluorohexane and perfluorocyclohexane.

37. A method of claim 20 wherein said ultrasound is applied at a frequency from about 40 kilohertz to about 25 megahertz, and an energy level of from about 500 milliwatts/cm^2 to about 10 watts/cm^2.

38. A method of claim 20 wherein said ultrasound is applied at a frequency of from about 500 kilohertz to about 200 kilohertz and said energy level is from about 500 milliwatts/cm^2 to about 200 milliwatts/cm^2.

39. A method of claim 20 wherein said ultrasound is applied at a frequency of from about 20 megahertz to about 1 megahertz and said energy level is from about 200 milliwatts/cm^2 to about 100 milliwatts/cm^2.

40. A method of claim 22 wherein said ultrasound is applied at a duty cycle of from about 1% to about 100% of the treatment time.

41. A method of claim 21 wherein said carrier is selected from the group consisting of polymers, lipids, proteins, and metal ions.

42. A method of claim 41 wherein said carrier is a protein.

43. A method of claim 41 wherein said protein is a cationic protein.

44. A method of claim 43 wherein said cationic protein is selected from the group consisting of polylysine and polyethyleneimine.

45. A method of claim 41 wherein said carrier is a lipid.

46. A method of claim 45 wherein said lipid is a cationic lipid.

47. A method of claim 46 wherein the carrier is a cationic lipid and said cationic lipid is N-[1(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride.

48. A method of claim 45 wherein said lipid is a fluorinated lipid.

49. A method of claim 48 wherein said fluorinated lipid is selected from compounds of the formula

$C_nF_{2n+1}(CH_2)_mC(O)OOP(OO^-)O(CH_2)_W\ N^+(CH_3)_3C_nF_{2n+1}(CH_2)_mC(O)O$

wherein: m is 0 to about 18, n is 1 to about 12; and w is 1 to about 8.

50. A method of claim 45 wherein said lipid is a fluorinated phospholipid.

51. A method of claim 41 wherein said carrier is a polymer.

52. A method of claim 51 wherein said polymer is selected from the group consisting of polyethylenes, polyoxyethylenes, polypropylenes, pluronic acids and alcohols, polyvinyls, polyvinylpyrrolidone, arabinans, fructans, fucans, galactans, galacturonans, glucans, mannans, xylans, levan, fucoidan, carrageenan, galactocarolose, pectin, pectic acid, amylose, pullulan, glycogen, amylopectin, cellulose, carboxylmethylcellulose, hydroxypropyl methylcellulose, dextran, pustulan, chitin, agarose, keratan, chondroitin, dermatan, hyaluronic acid, alginic acid, homopolymers and heteropolymers containing one or more of an aldose, ketose, acid, amine, erythrose, threose, ribose, arabinose, xylose, lyxose, allose, altrose, glucose, mannose, gulose, idose, galactose, talose, erythrulose, ribulose, xylulose, psicose, fructose, sorbose, tagatose, glucuronic acid, gluconic acid, glucaric acid, galacturonic acid, mannuronic acid, guluronic acid, glucosamine, galactosamine and neuraminic acid.

53. A method of claim 41 wherein said carrier is a metal ion.

54. A method of claim 41 wherein said carrier is a metal ion and said metal ion is selected from the group consisting of calcium ions, magnesium ions, and zinc ions.

55. A method of claim 21 wherein said carrier is selected from the group consisting of Lipofectin, Lipofectamine, Transfectace, Transfectam, Cytofectin, DMRIE, DLRIE, GAP-DLRIE, DOTAP, DOPE, DMEAP, DODMP, DOPC, DDAB, DOSPA, EDLPC, EDMPC,

DPH, TMADPH, CTAB, lysyl-PE, DC-Chol, -alanyl cholesterol, DCGS, DPPES, DCPE, DMAP, DMPE, DOGS, DOHME, DPEPC, Pluronic, Tween, BRIJ, plasmalogen, phosphatidylethanolamine, phosphatidylcholine, glycero-3-ethylphosphatidylcholine, dimethyl ammonium propane, trimethyl ammonium propane, diethylammonium propane, triethylammonium propane, dimethyldioctadecylammonium bromide, a sphingolipid, sphingomyelin, a lysolipid, a glycolipid, a sulfatide, a glycosphingolipid, cholesterol, cholesterol ester, cholesterol salt, oil, N-succinyldioleoylphosphatidylethanolamine, 1,2,-dioleoyl-sn-glycerol, 1,3-dipalmitoyl-2-succinylglycerol, 1,2-dipalmitoyl-sn-3-succinylglycerol, 1-hexadecyl-2-palmitoylglycerophosphatidylethanolamine, palmitoylhomocystiene, N,N'-Bis (dodecyaminocarbonylmethylene)-N,N'-bis ((-N,N,N-trimethylammoniumethyl-aminocarbonylmethylene)ethylenediamine tetra iodide; N,N''-Bis (hexadecylaminocarbonylmethylene)-N,N',N''-tris ((-N,N,N-trimethylammonium-ethylaminocarbonylmethylenediethylenetriamine hexaiodide; N,N'-Bis (dodecylaminocarbonylmethylene)-N,N"-bis((-N,N,N-trimethylammoniumethylaminocarbonylmethylene)cyclohexylene-1,4-diamine tetra iodide; 1,1,7,7-tetra-((-N,N,N,N-tetramethylammoniumethylamino-carbonylmethylene)-3-hexadecylaminocarbonyl-methylene-1,3,7-triaazaheptane heptaiodide; and N,N,N'N'-tetra((-N,N,N-trimethylammonium-ethylaminocarbonylmethylene)-N'-(1,2-dioleoylglycero-3-phosphoethanolaminocarbonylmethylene) diethylenetriamine tetra iodide.

56. A method of claim 21 wherein said carrier is selected from the group consisting of dioleoylphosphatidylethanolamine, a fatty acid, a lysolipid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, a sphingolipid, a glycolipid, a glucolipid, a sulfatide, a glycosphingolipid, phosphatidic acid, palmitic acid, stearic acid, arachidonic acid, oleic acid, a lipid bearing a polymer, a lipid bearing a sulfonated saccharide, cholesterol, tocopherol hemisuccinate, a lipid with an ether-linked fatty acid, a lipid with an ester-linked fatty acid, a polymerized lipid, diacetyl phosphate, stearylamine, cardiolipin, a phospholipid with a fatty acid of 6-8 carbons in length, a phospholipid with asymmetric acyl chains, 6-(5-cholesten-3b-yloxy)-1-

thio-b-D-galactopyranoside, digalactosyldiglyceride, 6-(5-cholesten-3b-yloxy)hexyl-6-amino-6-deoxy-1-thio-b-D-galactopyranoside, 6-(5-cholesten-3β-yloxy)hexyl-6-amino-6-deoxyl-1-thio-α-D-mannopyranoside,12-(((7'-diethylamino-coumarin-3-yl)carbonyl)methylamino)-octadecanoic acid; N-[12-(((7'-diethylaminocoumarin-3-yl)carbonyl)methyl-amino) octadecanoyl]-2-aminopalmitic acid; cholesteryl)4'-trimethyl-ammonio)butanoate; N-succinyldioleoyl-phosphatidylethanolamine; 1,2-dioleoyl-sn-glycerol; 1,2-dipalmitoyl-sn-3-succinyl-glycerol; 1,3-dipalmitoyl-2-succinylglycerol; 1-hexadecyl-2-palmitoylglycero-phosphoethanolamine, palmitoylhomocysteine, and/or combinations thereof.

57. A method of claim 56 wherein said carrier comprises phosphatidylcholine and said phosphatidylcholine is selected from the group consisting of dioleoylphosphatidylcholine, dimyristoylphosphatidylcholine, dipentadecanoylphosphatidylcholine, dilauroylphosphatidylcholine, dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine, and distearoylphosphatidylcholine.

58. A method of claim 56 wherein said carrier comprises phosphatidylethanolamine and said phosphatidylethanolamine is dioleoylphosphatidylethanolamine.

59. A method of claim 56 wherein said carrier comprises sphingolipid and said sphingolipid is sphingomyelin.

60. A method of claim 56 wherein said carrier comprises a glycolipid and said glycolipid is selected from ganglioside GM1 and ganglioside GM2.

61. A method of claim 56 wherein said carrier comprises a lipid bearing polymer and said polymer of said lipid bearing a polymer is selected from the group consisting of polyethylene glycol, chitin, hyaluronic acid, and polyvinylpyrrolidone.

62. A method of claim 61 wherein said polymer is polyethylethylene glycol and said polyethylene glycol is selected from the group consisting of polyethylene glycol having a molecular weight of about 2000, 5000, and 8000.

63. A method of claim 56 wherein said carrier comprises a lipid bearing a sulfonated saccharide.

64. A method of claim 56 wherein said carrier comprises cholesterol and said cholesterol is selected from the group consisting of cholesterol sulfate and cholesterol hemisuccinate.

65. A method of claim 56 wherein said carrier comprises a phospholipid with asymmetric acyl chains and said phospholipid with asymmetric acyl chains is a phospholipid having one acyl chain of about 6 carbons in length and another acyl chain of about 12 carbons in length.

66. A method of claim 21 wherein said carrier comprises about 82 mole percent dipalmitoylphosphatidylcholine, about 8 mole percent dipalmitoylphosphatidylethanolamine-polyethyleneglycol 5000 and about 10 mole percent dipalmitoylphosphatidic acid.

67. A method of claim 21 wherein said carrier is a surfactant.

68. A method of claim 67 where said surfactant is a fluorosurfactant.

69. A method of claim 20 wherein said organic halide is a gaseous precursor and wherein said gaseous precursor is converted to a gas after administration.

70. A method of claim 69 wherein said gaseous precursor is converted to a gas by applying ultrasound to said cell.

71. A method of claim 70 wherein said ultrasound is applied at a frequency from about from 40 kilohertz to about 25 megahertz, and said energy level is from about 500 milliwatts/cm^2 to about 10 watts/cm^2.

72. A method of claim 70 wherein said ultrasound is applied at a frequency of from about 500 kilohertz to about 200 kilohertz and said energy level is from about 500 milliwatts/cm^2 to about 200 milliwatts/cm^2.

73. A method of claim 70 wherein said ultrasound is applied at a frequency of from about 20 megahertz to about 1 megahertz and said energy level is from about 200 milliwatts/cm^2 to about 100 milliwatts/cm^2.

74. A method of claim 70 wherein said ultrasound is applied at a duty cycle of from about 1% to about 100% treatment time.

75. A method of claim 70 wherein said ultrasound is applied at a duty cycle of from about 10% to about 20%.

76. A method of claim 1 or 2 wherein said method is carried out *in vivo*.

77. A method of claim 1 or 2 wherein said method is carried out *ex vivo*.

78. A method of claim 1 or 2 wherein said method is carried out *in vitro*.

79. A method of claim 21 wherein said carrier comprises a vesicle.

80. A method of claim 79 wherein said sequence is encapsulated in said vesicle.

81. A method of identifying a nucleic acid sequence exhibiting increased synthesis comprising administering to a cell ultrasound for a time sufficient to increase synthesis of said nucleic acid sequence and observing an increase in synthesis of said nucleic acid sequence.

82. A method of claim 81 wherein observing said nucleic acid synthesis comprises amplifying said nucleic acid.

83. A method of claim 81 wherein said amplifying said nucleic acid comprises reverse transcriptase polymerase chain reaction.

84. A method of claim 82 wherein said observing said nucleic acid further comprises detecting said nucleic acid with a probe.

85. A method of claim 81 wherein said nucleic acid sequence is an endogenous sequence or an exogenous sequence.

86. A method of claim 81 wherein said sequence is an exogenous sequence and said exogenous sequence is provided simultaneously with said ultrasound, prior to said ultrasound, or after said ultrasound.

87. A method of claim 81 wherein said ultrasound is provided in a range of about 5 to about 40 kilohertz.

88. A method of claim 81 wherein said ultrasound is provided in a range of about 10 to about 20 kilohertz.

89. A method of claim 81 wherein said ultrasound is provided at about 25 kilohertz.

90. A method of claim 81 wherein said nucleic acid sequence is selected from the group consisting of DNA and RNA.

91. A method of claim 81 wherein said nucleic acid sequence is mRNA and observing said nucleic acid synthesis comprises reverse transcriptase polymerase chain reaction.

92. A method of claim 81 wherein said nucleic acid sequence is selected from the group consisting of initiation factor 3, tRNA synthetase, Ca+2 ATPase, ERCCl, Heme Oxygenase, Rad23, Raf, TCP-1-B, c-fos, calsequestrin, β-polymerase, 3-methyladenine DNA glycosylase, heat shock protein 27, heat shock protein 89α, MAP-kinase kinase, pericentrin, Ras, ubiquinone oxidoreductase complex, ubiquitin, XPA, XPB, XPG, B2, c-myc, c-jun, jun-b, Cox3, erg-1, HHR6B, HHR6A, RPA, SRC, catalase, and creatine.

93. A method of claim 81 wherein said cell is an animal cell or a plant cell.

94. A method of claim 93 wherein said animal cell is a mammalian cell.

95. A method of claim 93 wherein said mammalian cell is a human cell.

96. A method of claim 81 wherein said time sufficient to increase synthesis of said nucleic acid is from about 5 seconds to about 120 seconds.

97. A method of claim 81 wherein said time sufficient to increase synthesis of said nucleic acid is from about 30 seconds.

98. A method of claim 81 wherein said sequence is an exogenous sequence and said sequence is provided together with an organic halide.

99. A method of claim 98 wherein said composition further comprises a carrier.

100. A method of claim 98 wherein said organic halide is selected from the group consisting of a gaseous organic halide and a liquid organic halide.

101. A method of claim 100 wherein said organic halide is a gas.

102. A method of claim 100 wherein said organic halide is a liquid.

103. A method of claim 98 wherein said organic halide is a gaseous precursor.

104. A method of claim 98 wherein said organic halide is a fluorinated compound.

105. A method of claim 98 wherein said organic halide is a perfluorinated compound.

106. A method of claim 98 wherein said organic halide is a perfluorocarbon.

107. A method of claim 98 wherein said organic halide is a perfluoroether compound.

108. A method of claim 106 wherein said perfluorocarbon is a liquid.

109. A method of claim 108 wherein said liquid perfluorocarbon is a gaseous precursor.

110. A method of claim 106 wherein said perfluorocarbon is a gas.

111. A method of claim 98 wherein said organic halide is selected from the group consisting of 1-bromo-nonafluorobutane, perfluorooctyliodide, perfluoroocytlbromide, 1-chloro-1-fluoro-1-bromomethane, 1,1,1-trichloro-2,2,2-trifluoroethane, 1,2-dichloro-2,2-difluoroethane, 1,1-dichloro-1,2-difluoroethane, 1,2-dichloro-1,1,3-trifluoropropane, 1-bromoperfluorobutane, 1-bromo-2,4-difluorobenzene, 2-iodo-1,1,1-trifluoroethane, 5-bromovaleryl chloride, 1,3-dichlorotetrafluoroacetone, bromine pentafluoride, 1-bromo-1,1,2,3,3,3-hexafluoropropane, 2-chloro 1,1,1,4,4,4-hexafluoro-2-butene, 2-chloropentafluoro-1,3-butadiene, iodotrifluoroethylene, 1,1,2-trifluoro-2-chloroethane, 1,2-difluorochloroethane, 1,1-difluoro-2-chloroethane, 1,1-dichlorofluoroethane, heptafluoro-2-iodopropane, bromotrifluoroethane, chlorotrifluoromethane, dichlorodifluoromethane, dibromofluoromethane, chloropentafluoroethane,

bromochlorodifluoromethane, dichloro-1,1,2,2-tetrafluoroethane, 1,1,1,3,3-pentafluoropentane, perfluorotributylamine, perfluorotripropylamine, 3-fluorobenzaldehyde, 2-fluoro-5-nitrotoluene, 3-fluorostyrene, 3,5-difluoroaniline, 2,2,2-trifluoroethylacrylate, 3-(trifluoromethoxy)-acetophenone, 1,1,2,2,3,3,4,4-octafluorobutane, 1,1,1,3,3-pentafluorobutane, 1-fluorobutane, 1,1,2,2,3,3,4,4-octafluorobutane, 1,1,1,3,3-pentafluorobutane, perfluoro-4 methylquinolizidine, perfluoro-N-methyl-decahydroquinone, perfluoro-N-methyl-decahydroisoquinone, perfluoro-N-cyclohexyl-pyrrolidine, tetradecaperfluoroheptane, dodecaperfluorocyclohexane, perfluoromethane, perfluoroethane, perfluoropropane, perfluorobutane, perfluoropentane, perfluorohexane, perfluoroheptane, perfluorooctane, perfluorononane, perfluorodecane, perfluorododecane, perfluoro-2-methyl-2-pentene, perfluorocyclohexane, perfluorodecalin, perfluorododecalin, perfluoropropylene, perfluorocyclobutane, perfluoro-2-butyne, perfluoro-2-butene, perfluorobuta-1,3-diene, perfluorobutylethyl ether, bis(perfluoroisopropyl) ether, bis(perfluoropropyl) ether, perfluorotetrahydropyran, perfluoromethyl tetrahydrofuran, perfluoro t-butyl methyl ether, perfluoro isobutyl methyl ether, perfluoro n-butyl methyl ether, perfluoro isopropyl ethyl ether, perfluoro n-propyl ethyl ether, perfluoro cyclobutyl methyl ether, perfluoro cyclopropyl ethyl ether, perfluoro isopropyl methyl ether, perfluoro n-propyl methyl ether, perflouro diethyl ether, perfluoro cyclopropyl methyl ether, perfluoro methyl ethyl ether, perfluoro dimethyl ether, sulfur hexafluoride, and selenium hexafluoride.

112. A method of claim 98 wherein said organic halide is selected from the group consisting of perfluoromethane, perfluoroethane, perfluoropropane, perfluorobutane, perfluoropentane, perfluorohexane, perfluoroheptane, perfluorooctane, perfluorononane, perfluorodecane, perfluorododecane, perfluoro-2-methyl-2-pentene, perfluorocyclohexane, perfluorodecalin, perfluorododecalin, tetradecaperfluorohexane, and dodecaperfluorocyclohexane.

113. A method of claim 98 wherein said organic halide is selected from the group consisting of perfluorobutylethyl ether, bis(perfluoroisopropyl) ether, bis(perfluoropropyl) ether, perfluorotetrahydropyran, perfluoromethyl tetrahydrofuran, perfluoro t-butyl methyl

ether, perfluoro isobutyl methyl ether, perfluoro n-butyl methyl ether, perfluoro isopropyl ethyl ether, perfluoro n-propyl ethyl ether, perfluoro cyclobutyl methyl ether, perfluoro cyclopropyl ethyl ether, perfluoro isopropyl methyl ether, perfluoro n-propyl methyl ether, perflouro diethyl ether, perfluoro cyclopropyl methyl ether, perfluoro methyl ethyl ether, and perfluoro dimethyl ether.

114. A method of claim 98 wherein said organic halide is selected from the group consisting of perfluorohexane and perfluorocyclohexane.

115. A method of claim 98 wherein said ultrasound is applied at a frequency from about 40 kilohertz to about 25 megahertz, and an energy level of from about 500 milliwatts/cm^2 to about 10 watts/cm^2.

116. A method of claim 98 wherein said ultrasound is applied at a frequency of from about 500 kilohertz to about 200 kilohertz and said energy level is from about 500 milliwatts/cm^2 to about 200 milliwatts/cm^2.

117. A method of claim 98 wherein said ultrasound is applied at a frequency of from about 20 megahertz to about 1 megahertz and said energy level is from about 200 milliwatts/cm^2 to about 100 milliwatts/cm^2.

118. A method of claim 100 wherein said ultrasound is applied at a duty cycle of from about 1% to about 100% of the treatment time.

119. A method of claim 99 wherein said carrier is selected from the group consisting of polymers, lipids, proteins, and metal ions.

120. A method of claim 119 wherein said carrier is a protein.

121. A method of claim 119 wherein said protein is a cationic protein.

122. A method of claim 121 wherein said cationic protein is selected from the group consisting of polylysine and polyethyleneimine.

123. A method of claim 119 wherein said carrier is a lipid.

124. A method of claim 123 wherein said lipid is a cationic lipid.

125. A method of claim 124 wherein the carrier is a cationic lipid and said cationic lipid is N-[1(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride.

126. A method of claim 122 wherein said lipid is a fluorinated lipid.

127. A method of claim 126 wherein said fluorinated lipid is selected from compounds of the formula

$C_nF_{2n+1}(CH_2)_mC(O)OOP(OO^-)O(CH_2)_W\ N^+(CH_3)_3C_nF_{2n+1}(CH_2)_mC(O)O$

wherein: m is 0 to about 18, n is 1 to about 12; and w is 1 to about 8.

128. A method of claim 123 wherein said lipid is a fluorinated phospholipid.

129. A method of claim 119 wherein said carrier is a polymer.

130. A method of claim 129 wherein said polymer is selected from the group consisting of polyethylenes, polyoxyethylenes, polypropylenes, pluronic acids and alcohols, polyvinyls, polyvinylpyrrolidone, arabinans, fructans, fucans, galactans, galacturonans, glucans, mannans, xylans, levan, fucoidan, carrageenan, galactocarolose, pectin, pectic acid, amylose, pullulan, glycogen, amylopectin, cellulose, carboxylmethylcellulose, hydroxypropyl methylcellulose, dextran, pustulan, chitin, agarose, keratan, chondroitin, dermatan, hyaluronic acid, alginic acid, homopolymers and heteropolymers containing one or more of an aldose, ketose, acid, amine, erythrose, threose, ribose, arabinose, xylose,

lyxose, allose, altrose, glucose, mannose, gulose, idose, galactose, talose, erythrulose, ribulose, xylulose, psicose, fructose, sorbose, tagatose, glucuronic acid, gluconic acid, glucaric acid, galacturonic acid, mannuronic acid, guluronic acid, glucosamine, galactosamine and neuraminic acid.

131. A method of claim 119 wherein said carrier is a metal ion.

132. A method of claim 119 wherein said carrier is a metal ion and said metal ion is selected from the group consisting of calcium ions, magnesium ions, and zinc ions.

133. A method of claim 99 wherein said carrier is selected from the group consisting of Lipofectin, Lipofectamine, Transfectace, Transfectam, Cytofectin, DMRIE, DLRIE, GAP-DLRIE, DOTAP, DOPE, DMEAP, DODMP, DOPC, DDAB, DOSPA, EDLPC, EDMPC, DPH, TMADPH, CTAB, lysyl-PE, DC-Chol, -alanyl cholesterol, DCGS, DPPES, DCPE, DMAP, DMPE, DOGS, DOHME, DPEPC, Pluronic, Tween, BRIJ, plasmalogen, phosphatidylethanolamine, phosphatidylcholine, glycero-3-ethylphosphatidylcholine, dimethyl ammonium propane, trimethyl ammonium propane, diethylammonium propane, triethylammonium propane, dimethyldioctadecylammonium bromide. a sphingolipid, sphingomyelin. a lysolipid, a glycolipid. a sulfatide, a glycosphingolipid, cholesterol, cholesterol ester, cholesterol salt, oil, N-succinyldioleoylphosphatidylethanolamine, 1,2,-dioleoyl-sn-glycerol, 1,3-dipalmitoyl-2-succinylglycerol, 1,2-dipalmitoyl-sn-3-succinylglycerol, 1-hexadecyl-2-palmitoylglycerophosphatidylethanolamine, palmitoylhomocystiene, N,N'-Bis (dodecyaminocarbonylmethylene)-N,N''-bis ((-N,N,N-trimethylammoniumethyl-aminocarbonylmethylene)ethylenediamine tetraiodide; N,N''-Bis (hexadecylaminocarbonylmethylene)-N,N',N''-tris ((-N,N,N-trimethylammonium-ethylaminocarbonylmethylenediethylenetriamine hexaiodide; N,N'-Bis (dodecylaminocarbonylmethylene)-N,N"-bis((-N,N,N-trimethylammoniumethylaminocarbonylmethylene)cyclohexylene-1,4-diamine tetraiodide; 1,1,7,7-tetra-((-N,N,N,N-tetramethylammoniumethylamino-carbonylmethylene)-3-hexadecylaminocarbonyl-methylene-1,3,7-triaazaheptane heptaiodide;

and N,N,N'N'-tetra((-N,N,N-trimethylammonium-ethylaminocarbonylmethylene)-N'-(1,2-dioleoylglycero-3-phosphoethanolaminocarbonylmethylene) diethylenetriamine tetraiodide.

134. A method of claim 99 wherein said carrier is selected from the group consisting of dioleoylphosphatidylethanolamine, a fatty acid, a lysolipid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, a sphingolipid, a glycolipid, a glucolipid, a sulfatide, a glycosphingolipid, phosphatidic acid, palmitic acid, stearic acid, arachidonic acid, oleic acid, a lipid bearing a polymer, a lipid bearing a sulfonated saccharide, cholesterol, tocopherol hemisuccinate, a lipid with an ether-linked fatty acid, a lipid with an ester-linked fatty acid, a polymerized lipid, diacetyl phosphate, stearylamine, cardiolipin, a phospholipid with a fatty acid of 6-8 carbons in length, a phospholipid with asymmetric acyl chains, 6-(5-cholesten-3b-yloxy)-1- thio-b-D-galactopyranoside, digalactosyldiglyceride, 6-(5-cholesten-3b-yloxy)hexyl- 6-amino-6-deoxy-1-thio-b-D-galactopyranoside, 6-(5-cholesten-3β-yloxy)hexyl-6-amino-6-deoxyl-1-thio-α-D-mannopyranoside,12-(((7'-diethylamino-coumarin-3-yl)carbonyl)methylamino)-octadecanoic acid; N-[12-(((7'-diethylaminocoumarin-3-yl)carbonyl)methyl-amino) octadecanoyl]-2-aminopalmitic acid; cholesteryl)4'-trimethyl-ammonio)butanoate; N-succinyldioleoyl-phosphatidylethanolamine; 1,2-dioleoyl-sn-glycerol; 1,2-dipalmitoyl-sn-3-succinyl-glycerol; 1,3-dipalmitoyl-2-succinylglycerol; 1-hexadecyl-2-palmitoylglycero-phosphoethanolamine, palmitoylhomocysteine, and/or combinations thereof.

135. A method of claim 134 wherein said carrier comprises phosphatidylcholine and said phosphatidylcholine is selected from the group consisting of dioleoylphosphatidylcholine, dimyristoylphosphatidylcholine, dipentadecanoylphosphatidylcholine, dilauroylphosphatidylcholine, dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine, and distearoylphosphatidylcholine.

136. A method of claim 134 wherein said carrier comprises phosphatidylethanolamine and said phosphatidylethanolamine is dioleoylphosphatidylethanolamine.

137. A method of claim 134 wherein said carrier comprises sphingolipid and said sphingolipid is sphingomyelin.

138. A method of claim 134 wherein said carrier comprises a glycolipid and said glycolipid is selected from ganglioside GM1 and ganglioside GM2.

139. A method of claim 134 wherein said carrier comprises a lipid bearing polymer and said polymer of said lipid bearing a polymer is selected from the group consisting of polyethylene glycol, chitin, hyaluronic acid, and polyvinylpyrrolidone.

140. A method of claim 139 wherein said polymer is polyethylethylene glycol and said polyethylene glycol is selected from the group consisting of polyethylene glycol having a molecular weight of about 2000, 5000, and 8000.

141. A method of claim 134 wherein said carrier comprises a lipid bearing a sulfonated saccharide.

142. A method of claim 134 wherein said carrier comprises cholesterol and said cholesterol is selected from the group consisting of cholesterol sulfate and cholesterol hemisuccinate.

143. A method of claim 134 wherein said carrier comprises a phospholipid with asymmetric acyl chains and said phospholipid with asymmetric acyl chains is a phospholipid having one acyl chain of about 6 carbons in length and another acyl chain of about 12 carbons in length.

144. A method of claim 99 wherein said carrier comprises about 82 mole percent dipalmitoylphosphatidylcholine, about 8 mole percent dipalmitoylphosphatidylethanolamine-polyethyleneglycol 5000 and about 10 mole percent dipalmitoylphosphatidic acid.

145. A method of claim 99 wherein said carrier is a surfactant.

146. A method of claim 145 where said surfactant is a fluorosurfactant.

147. A method of claim 99 wherein said organic halide is a gaseous precursor and wherein said gaseous precursor is converted to a gas after administration.

148. A method of claim 147 wherein said gaseous precursor is converted to a gas by applying heat to said cell.

149. A method of claim 148 wherein said heat is applied by ultrasound.

150. A method of claim 149 wherein said ultrasound is applied at a frequency from about from 40 kilohertz to about 25 megahertz, and said energy level is from about 500 milliwatts/cm^2 to about 10 watts/cm^2.

151. A method of claim 149 wherein said ultrasound is applied at a frequency of from about 500 kilohertz to about 200 kilohertz and said energy level is from about 500 milliwatts/cm^2 to about 200 milliwatts/cm^2.

152. A method of claim 149 wherein said ultrasound is applied at a frequency of from about 20 megahertz to about 1 megahertz and said energy level is from about 200 milliwatts/cm^2 to about 100 milliwatts/cm^2.

153. A method of claim 149 wherein said ultrasound is applied at a duty cycle of from about 1% to about 100% treatment time.

154. A method of claim 149 wherein said ultrasound is applied at a duty cycle of from about 10% to about 20%.

155. A method of claim 81 wherein said method is carried out *ex vivo*.

156. A method of claim 81 wherein said method is carried out *in vitro*.

157. A method of claim 99 wherein said carrier comprises a vesicle.

158. A method of claim 157 wherein said sequence is encapsulated in said vesicle.

159. A method of treating a condition in a human subject comprising administering to the subject a therapeutically effective amount of ultrasound for a therapeutically effective amount of time such that said ultrasound increases synthesis of a nucleic acid sequence in said subject, wherein said increase in synthesis of said nucleic acid sequence results in treating said subject.

160. A method of claim 159 wherein ultrasound is delivered to said subject from a portable ultrasound device.

161. A method of claim 160 wherein said subject wears said portable ultrasound device.

162. A method of claim 159 wherein said device is manually activated.

163. A method of claim 159 wherein said device is automatically activated at predetermined intervals.

164. A method of claim 159 wherein said nucleic acid sequence is endogenous or exogenous.

165. A method of treating a human subject suspected of having phenylketonuria comprising administering to said human subject an exogenous nucleic acid sequence encoding phenylalanine hydroxylase and a therapeutically effective amount of ultrasound for a therapeutically effective amount of time such that said ultrasound increases synthesis of said phenylalanine hydroxylase nucleic acid sequence.

166. A method of claim 165 for treating phenylketonuria wherein said exogenous nucleic acid sequence is administered in a solid porous matrix of 1:3 DPEPC: 3 dioleoylphosphatidylethanolamine (DOPE) and perfluorohexane, said matrix comprising a plasmid having a phenylalanine hydroxylase nucleic acid sequence.

167. A method for increasing synthesis of a nucleic acid sequence encoding tumor suppressor gene p53 in a human subject comprising administering to said human subject an exogenous nucleic acid sequence encoding p53 and a therapeutically effective amount of ultrasound for a therapeutically effective amount of time such that said ultrasound increases synthesis of said p53 nucleic acid sequence.

168. A method of claim 167 wherein said exogenous nucleic acid sequence is administered in a solid porous matrix of 1:3 DPEPC: dioleoylphosphatidylethanolamine (DOPE) and perfluorohexane. said matrix comprising a plasmid encoding p53.

169. A method for increasing synthesis of a nucleic acid sequence encoding IL-2 in a human subject comprising administering to said human subject an exogenous nucleic acid sequence encoding IL-2 and a therapeutically effective amount of ultrasound for a therapeutically effective amount of time such that said ultrasound increases synthesis of said IL-2 nucleic acid sequence.

170. A method of claim 169 wherein said IL-2 is associated with killer T lymphocytes.

171. A method of claim 169 wherein said exogenous nucleotide sequence is administered in a solid porous matrix of DMRIE/DOPE, said matrix comprising a plasmid encoding IL-2.

172. A method of effecting a change in the expression of an endogenous nucleotide sequence in a cell comprising administering to said cell a therapeutically effective amount of ultrasound for a therapeutically effective time such that said administration of said ultrasound results in said change in expression of said nucleic acid sequence, wherein said endogenous nucleic acid sequence encoding a protein selected from the group consisting of a stress protein and a cellular repair protein.

173. A method of effecting the expression of an exogenous nucleotide sequence in a cell comprising administering to the cell a nucleic acid sequence and applying a therapeutically effective amount of ultrasound for a therapeutically effective time such that said administration of said ultrasound results in expression of said nucleic acid sequence.

174. A method of treating cancer comprising administering to a human subject an exogenous antisense sequence of IF3, tRNA synthetase, or a combination thereof, and a therapeutically effective amount of ultrasound for a therapeutically effective amount of time such that ultrasound increases synthesis of said antisense sequence, thereby treating cancer.

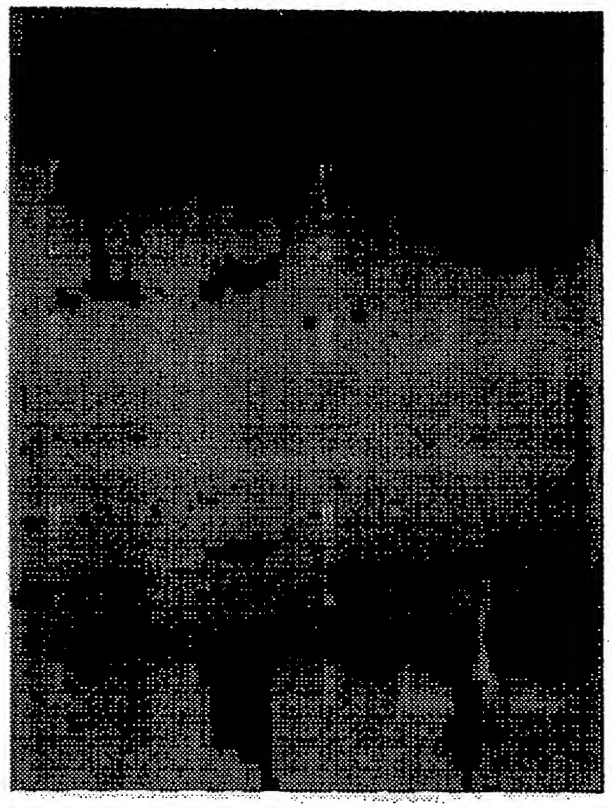

FIGURE 1

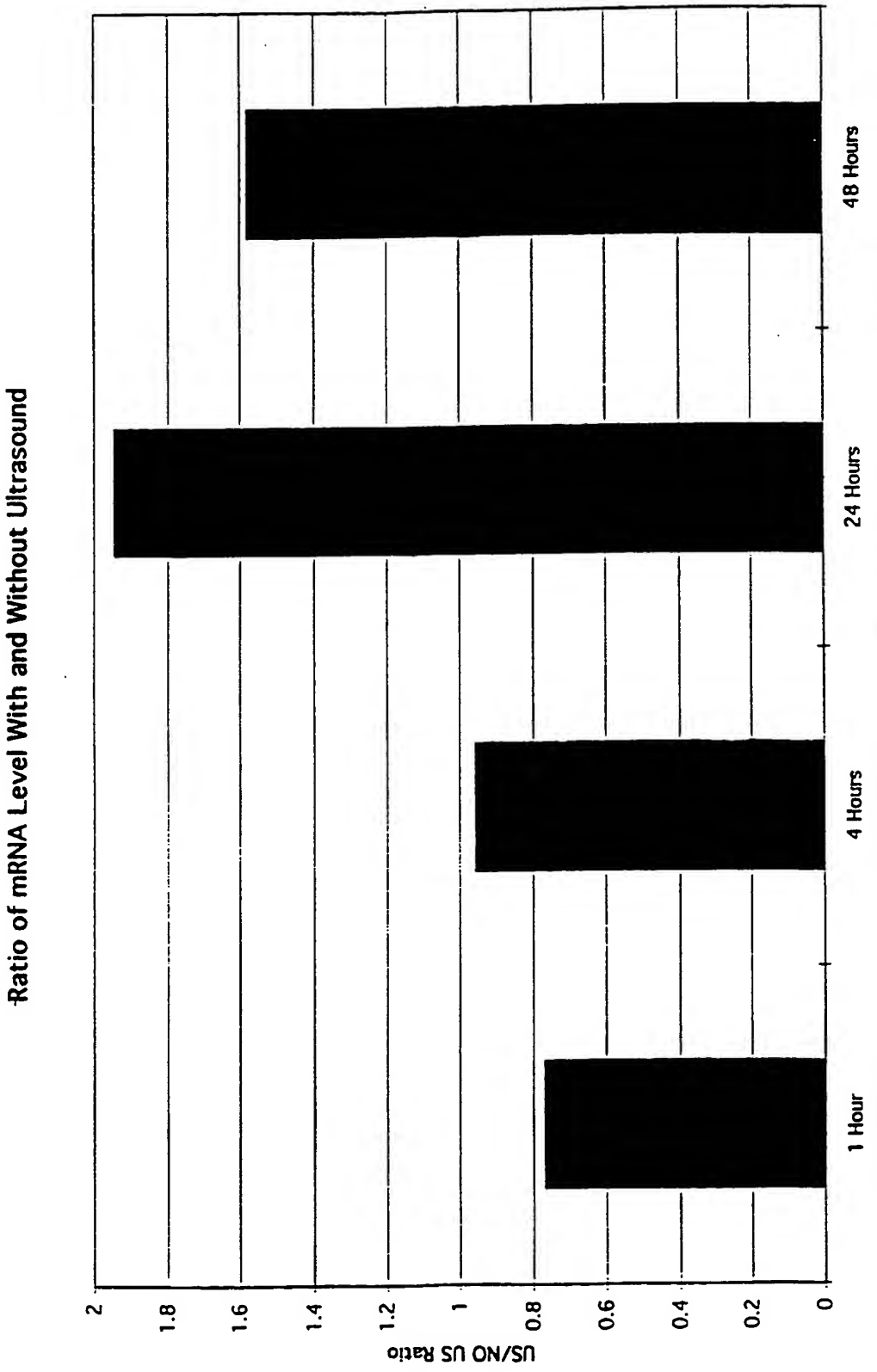
Ratio of mRNA Level With and Without Ultrasound
US/NO US Ratio
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1 Hour
4 Hours
24 Hours
48 Hours
Time

FIGURE 2

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANTS: IMARx PHARMACEUTICAL CORP.

(ii) TITLE OF INVENTION: A Method of Increasing Nucleic Acid Synthesis With Ultrasound

(iii) NUMBER OF SEQUENCES: 73

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE:Woodcock Washburn Kurtz Mackiewicz & Norris LLP

(B) STREET: One Liberty Place - 46th Floor

(C) CITY: Philadelphia

(D) STATE: PA

(E) COUNTRY: USA

(F) ZIP: 19103

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: DISKETTE, 3.5 INCH, 1.44 Mb STORAGE

(B) COMPUTER: IBM PS/2

(C) OPERATING SYSTEM: PC-DOS

(D) SOFTWARE: WORDPERFECT for WINDOWS 6.0

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: unknown

(B) FILING DATE: 10 NOV 98

(C) CLASSIFICATION: unknown

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: U.S. Serial No. 08/971,540

(B) FILING DATE: 17 NOV 97

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Lori Y. Beardell

(B) REGISTRATION NUMBER: 34,293

(C) REFERENCE/DOCKET NUMBER: UNGR-1526

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (215) 568-3100

(B) TELEFAX: (215) 568-3439

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1

GGCCACAAAC CCACTGATGA A 21

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2

GGCCACCACT CGGACAAAAA C 21

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3

GCCCTCCCAT CCCTGTGA 18

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY:unknown

(xi)SEQUENCE DESCRIPTION: SEQ ID NO: 4

CATCCAATCC TTCCCGTGCT A 21

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi)SEQUENCE DESCRIPTION: SEQ ID NO: 5

TACNCAAGCT TGGCACGAAG G 21

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi)SEQUENCE DESCRIPTION: SEQ ID NO: 6

AAATCAACGG CAGCACCAG 19

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7

TGCTCATGGT GGGGGTTTA 19

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8

AGTGCTGATG AATGGGGAGA G 21

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9

AATGGCCTTG TGAAAA 16

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10

AAAAATTATA TTGGCATCTT C 21

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11

CACGCTATGG CTGACA 16

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12

AACTTGCCTT CTAATCATTT T 21

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13

TCATTTTACC GCCAACCATC C 21

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14

TTTCTTTTCC GACCGCTTCA G 21

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15

CACATATGTG GGGCAGAGC 19

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16

ACATAGAAGC GGAGGGGTTT C 21

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17

GGCTGAAGAA GTATGACAA 19

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18

AAGAAGTAGT AAGGGGGAAA T 21

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19

ACCACCTCCT TCCCCACCAG T 21

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20

GCCCAGCAGC CAGATGTTCA C 21

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21

GGAACCAGGG GAGATG 16

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22

TATAAATTGG GAAAGGGAAA G 21

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23

GGGCCGCCAG CAAGGAAGAA 20

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24

TTGGCAGCTG GGGTCATCAG G 21

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25

TCAGCCAGTC GCCAAGAATC A 21

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26

GCCCAGGAAT GCCAACCAAT C 21

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27

CCGCCGAGCC TAAACTA 17

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28

CATAAGCTAT TCTGCCACCT C 21

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29

TTCTAGATCC TGAGCCCTGA C 21

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30

GGGGATGGGT AGCAAGC 17

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31

AAATGCTTCG GTTACCCACA G 21

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32

TGGCAAGCAC TAAAATCCTG A 21

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33

GTTCCTCTGG GTGCTTCC 18

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34

AGATCGCAGA CATTGGCTTT A 21

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35

CAGAGGGGGT AGACGAGTCA 20

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36

TAGTACCAGG CCAAGAGTCC A 21

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37

TTGGGGGTGC TGTATGCT 18

(2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38

AGATGGGCTT TGTTTTGTTG A 21

(2) INFORMATION FOR SEQ ID NO: 39:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39

CGTGCGTTGG GTTCTT 16

(2) INFORMATION FOR SEQ ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40

AAGTGTGTAA GTGCCGTGTT A 21

(2) INFORMATION FOR SEQ ID NO: 41:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41

TAACTTCTAC CGCTCCTTCC A 21

(2) INFORMATION FOR SEQ ID NO: 42:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42

CCACTAACGC CCTCCTGT 18

(2) INFORMATION FOR SEQ ID NO: 43:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43

CAGCGGCAGA TGACTGAG 18

(2) INFORMATION FOR SEQ ID NO: 44:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44

CTTATAACTG CCCGACCAAG A 21

(2) INFORMATION FOR SEQ ID NO: 45:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45

CCCCGTGGCT GGACAGAAAC T 21

(2) INFORMATION FOR SEQ ID NO: 46:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46

TGGGGGTGGC CTTGACAATG 20

(2) INFORMATION FOR SEQ ID NO: 47:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 47

CCCCCAGACC CAGAACTCCA T 21

(2) INFORMATION FOR SEQ ID NO: 48:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 48

CTCCCACGCC TCCACCTTGT 20

(2) INFORMATION FOR SEQ ID NO: 49:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 49

CCGCAGTCTT GGACCAT 17

(2) INFORMATION FOR SEQ ID NO: 50:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 50

TATAAAAGGG CTTCGGTGAG A 21

(2) INFORMATION FOR SEQ ID NO: 51:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 51

CTTGGGGGTT GGATTCTCTT C 21

(2) INFORMATION FOR SEQ ID NO: 52:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 52

GGGGCAGGCG ATGGTAG 17

(2) INFORMATION FOR SEQ ID NO: 53:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 53

ACGCCCCTTT GACGCTCCTA C 21

(2) INFORMATION FOR SEQ ID NO: 54:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 54

GTTGCCTCAA GCCCCACCTT T 21

(2) INFORMATION FOR SEQ ID NO: 55:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 55

CAGAGCGGAG CTATCGGTTG T 21

(2) INFORMATION FOR SEQ ID NO: 56:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 56

ACGGCATTGC ACCTGACACT 20

(2) INFORMATION FOR SEQ ID NO: 57:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 57

ATGGCGGGTG TGCTGA 16

(2) INFORMATION FOR SEQ ID NO: 58:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 58

GCCAACTTCT TTCCCTGAAC A 21

(2) INFORMATION FOR SEQ ID NO: 59:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 59

AGCCGCAGCC AACCAGTGT 19

(2) INFORMATION FOR SEQ ID NO: 60:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 60

TTCCCTACCC ACCCCGAAGA C 21

(2) INFORMATION FOR SEQ ID NO: 61:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 61

TGTACAGACG CTGGCTATGC T 21

(2) INFORMATION FOR SEQ ID NO: 62:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 62

GGGAGAGGGA AGGGGAAAG 19

(2) INFORMATION FOR SEQ ID NO: 63:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 63

GACTTTCTTA TCGCCATTGC T 21

(2) INFORMATION FOR SEQ ID NO: 64:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 64

CCCCACTCTC TGTTTCTCAT 20

(2) INFORMATION FOR SEQ ID NO: 65:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 65

TTTCGTATCC GCCCTATTTT T 21

(2) INFORMATION FOR SEQ ID NO: 66:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 nucleic acids

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 66

CCCACCTTCT GTTTCATCGT 20

(2) INFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 146 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

CCTGGGCCCC CATGCCCGTG CAGCTCGCAC ATATGTGGGG CAGAGCAGCC ACCCTGCCCC 60

CAGCAGCAGC CGTCCATCGT CAGACGTGAT CATTTCCTGA GGCCTCGAGT GTGTCAGGGT 120

GTTTGTGCCT CATAACAACC CACAGG 146

(2) INFORMATION FOR SEQ ID NO:68:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 257 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

GGATCCTGAG CTTCAAGAGC AAGAAGAGAT TATCTAATGG AAATGTTGTA CTTGTGTATA 60

AATGATGAAA AGGATGCCCC ATTTCCTTTG AGACACCCTC CTTACGTTTT TGACCGCCCG 120

TGCCAGTTTG ATATGGGCGG TCTTTATGGT TGTGACGCCT TTCTTTTCTC TAAAAACGCT 180

TTGCCATAGC TGCCGTGGTC ATGAAGTATG GCGTGTTCCA CTTGAATGGT GGAGTGCTCG 240

ATGCCGTATT TTTCCTT 257

(2) INFORMATION FOR SEQ ID NO:69:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1509 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

TACNCAAGCT TGGCACGAAG GCTGTCCCTT GGCTTCTGTG TGGTCCCAAA AAAATATGCC 60

TCCTCCAGTT TCAAGGCTGC AGACCTGCAG CTGGAAATGA CACAGAAGCC TCATAAGAAG 120

CCTGGCCCCG GCGAGCCCCT GGTGTTTGGG AAAACATTTA CCGACCACAT GCTGATGGTG 180

GAATGGAATG ACAAGGGCTG GGGCCAGCCC CGAATCCAGC CCTTCCAGAA CCTCACGCTG 240

CACCCAGCCT CCTCCAGCCT CCACTACTCC CTGCAGCTGT TTGAGGGCAT GAAGGCGTTC 300

AAAGGCAAAG ACCAGCAGGT GCGCCTCTTC CGCCCCTGGC TCAACATGGA CCGGATGCTG 360

CGCTCAGCCA TGCGCCTGTG CCTGCCGAGT TTCGACAAGC TGGAGTTGCT GGAGTGCATC 420

CGCCGGCTCA TCGAATTGGA CAAGGACTGG GTCCCCGATG CCGCCGGCAC CAGCCTCTAT 480

GTGCGGCCTG TGCTCATTGG GAACGAGCCC TCGCTGGGTG TCAGCCAGCC CAGGCGCGCG 540

CTCCTGTTCG TCATTCTCTG CCCAGTGGGT GCCTACTTCC CTGGAGGCTC CGTGAACCCG 600

GTCTCCCTCC TGGCCGAACC AACCTTCATC CGGGCCTGGG TTGGCGGGGT CGGCAACTAC 660

AAGTTAGGTG GGAATTATGG GCCCACCGTG TTAGTGCAAC AGGAGGCACT CAAGCGGGGC 720

TGTGAACAGG TCTTCTGGCT GTATGGGCCC GACCACCAGC TCACCGAGGT GGGAACCATG 780

AACATCTTTG TCTACTGGAC CCACGAAGAT GGGGTGCTGG AGCTGGTGAC GCCCCCGCTG 840

AATGGTGTTA TCCTGCCTGG AGTGGTCAGA CAGAGTCTAC TGGACATGGC TCAGACCTGG 900

GGTGAGTTCC GGGTGGTGGA GCGCACGATC ACCATGAAGC AGTTGTTGCG GCCCTTGGAG 960

GAGGCCCGCG TGCGGGAAGT CTTTGGCTCG GGCACCGCTT GCCAGGTCTG CCCAGTGCAC 1020

GGAATCCTGT ACAAAGACAG GAACTTCCAT ATTCCCACCA TGGAAAATGG GCCTGAGCTG 1080

ATCTTCCGCT TCCAGAAGGA GCTGAAGGAG ATCCAGTACG GAATCAGAGC CCACGAGTGG 1140

ATGTTCCCGG TGTGAAGCTG CAGGCTGTGC TCCAGATCCA CCGACCCGTA GCATNTCGTA 1200

ACGCCAGCAC TCGCNTCCTT ACCAATGACT CACCTGAAGT GCAATACGAA ATAAAAGGCC 1260

AGCGGGCGGC GTCTGGGTCT CTGGCGCCCC CATGTGGTTG CGACACTCCC AAAGCCGTAA 1320

GGGCCGACCC AGGCATCTTG GCCCCCAGCC CNTCGTCGCG GGTTCAGGTC CGCCCATTAC 1380

TCCCTTGTCG TGCGGTCAAG GATACACCTT GGCCCCGATT CCGGATCTCT CCGTTCTCAG 1440

GCCAGACCCC TGGTGCTGCC GTTGATTTTT TTTTCTCTGT CTTTGCTGCA ATTTTGAAAT 1500

AAAATGCCA 1509

(2) INFORMATION FOR SEQ ID NO:70:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 566 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

GCTCCTTTCT TTAGAGTTGT GAAAAAGATA CAGAAGTTGA TCAAGCCGGG TGACAGAGTA 60

AGACCCTGTA TCATTATTAT TTTATTTTGA AACAGAGTCC CACTCTGTCG CCCAGGCTGG 120

AGTGCAGTGG CATAATCTCG GCTCACTACA ACCTCCGCCT CCTGGGTTCC AGCGAGTCTC 180

CTGCCTCAGC CTCCTGAGTA GCTGGGGTAT TACAGGTGCG CGCCACCACA CCTGGCTAAT 240

TTTTCATTTT TAGTAGAGAC GGGATTTCAC CATGTTGGTC AGGCTGGTCT CGAACTCCTG 300

ACCTCGTGAT CTGCCCACCA CGGCCTCCCA AAGTGCTGGG ATTACAGGCG TGAGCCACTG 360

CGCCCGGCCC CCTGTATCCT TTTTTTTTTT TTTTTTTTGG AGACGAGTCT CACTCTGTCG 420

CCAGGCTTGA GTGCAGTGGT GCGATCTCGG CTCACTGCAA CCTCCGCCTC CCAGGTTCAA 480

GCAATTCTCC TGTCTCAGCC TCCCGAGTAG CTGGGATTAC AGGCGAACGC TACCATGCCC 540

GGCTAATTTT TGTATTTTTA GTAGAG 566

(2) INFORMATION FOR SEQ ID NO:71:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 147 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

GCGGTTCCTC TGGGTGCTTC CGCCTCCCCT TCTCCTGCTT CTCCAGCCTC TTCGGCCTCC 60

TCGCCCGCCG CGGGAACCCG AGACCCCAGT GTATGCCCCA CCCCTGACCC CGCTCGCGAC 120

ATGTCCACCC CGGCTCGGCG GCGCCTC 147

(2) INFORMATION FOR SEQ ID NO:72:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 141 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

CACGCTTGCC GCCGCCCCGC AGAAATGCTT CGGTTACCCA CAGTCTTTCG CCAGATGAGA 60

CCGGTGTCCA GGGTACTGGC TCCTCATCTC ACTCGGGCTT ATGCCAAAGA TGTAAAATTT 60

GGTGCAGATG CCCGAGCCTT A 21

(2) INFORMATION FOR SEQ ID NO:73:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 146 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

GCTGCTTGCC TTTGACCTGC GAGATCGCTT TGTTTTTTGG CAGTGCCGTG TTAGTGTGCC 60

TCGCCCTGTA TGGTTATCGA TTTCTTTTTA TTTGCCTTAC TCCCGATCTT GCTCGCTGGG 120

GTGCCGCCGC CTAACCCCGG CTCTTT 146

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/23843

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 48/00; A61H 1/00
US CL :514/44; 601/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 601/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, EMBASE, MEDLINE, DERWENT BIOTECHNOLOGY, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	US 4,530,360 A (DUARTE) 23 July 1985, see the entire document.	1, 3, 4, 12-15,19,76,159,164,172 ------- 9-11, 15-18,77,78,159-164
Y	US 5,556,372 A (TALISH et al) 17 September 1996, see the entire document.	159-164
Y	US 5,571,797 A (OHNO et al) 05 November 1996, see the entire document.	2, 5-8, 20-75,79,80,165-171,173,174
Y	US 5,612,318 A (WEICHSELBAUM et al) 18 March 1997, see the entire document.	2, 5-8, 20-75, 79, 80, 165-171,173,174

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
01 FEBRUARY 1999

Date of mailing of the international search report
25 FEB 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
BRUCE CAMPELL
Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/23843

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WANG et al. Low Intensity Ultrasound Treatment Increases Strength in a Rat Femoral Fracture Model. Journal of Orthopaedic Research. 1994, Vol. 12, No. 1, pages 40-47, see the entire document.	1,3,4,12-14,19,76,172 -------- 9-11,15-18,77,78
X - Y	JACKSON et al. Effect of ultrasound therapy on the repair of Achilles tendon injuries in rats. Medicine and Science in Sports and Exercise. 1991, Vol. 23, No. 2, pages 171-176, see the entire document.	1,3,4,12-14,19,76,172 -------- 9-11,15-18,77,78
X - Y	YOUNG et al. The Effect of Therapeutic Ultrasound on Angiogenesis. Ultrasound in Medicine and Biology. 1990, Vol. 16, No. 3, pages 261-269, see the entire document.	1,3,4,12-14,19,76,172 -------- 9-11,15-18,77,78
X - Y	YOUNG et al. Effect of therapeutic ultrasound on the healing of full-thickness excised skin lesions. Ultrasonics. 1990, Vol. 28, No. 3, pages 175-180, see the entire document.	1,3,4,12-14,19,76,172 -------- 9-11,15-18,77,78
Y	MAXWELL, L. Therapeutic Ultrasound: Its Effects on the Cellular and Molecular Mechanisms of Inflammation and Repair. Physiotherapy. June 1992, Vol. 78, No. 6, pages 421-426, see page 421, column 2.	2,5-8,20-75,79,80,165-171,173,174
Y	TUNCAY et al. Expression of Genes Associated with Tissue Remodeling Upon Ultrasound Perturbation in the Gingival Fibroblast. Journal of Dental Research. 1996, Vol. 75, special issue, page 143, #1007, see the entire abstract.	2,5-8,20-75,79,80,165-171,173,174
Y	YANG et al. Exposure to Low-Intensity Ultrasound Increases Aggrecan Gene Expression in a Rat Femur Fracture Model. Journal of Orthopaedic Research. 1996, Vol. 14, pages 802-809, see the entire document.	2,5-8,20-75,79,80,165,171,173,174
Y	Database BIOSIS, No. 1993: 95122245, LEJBKOWICZ et al. The response of normal and malignant cells to ultrasound in vitro. Ultrasound in Medicine and Biology. 1993, Vol. 19, No. 1, pages 75-82 (abstract only), see the entire abstract.	77,78

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/23843

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-80 and 159-174

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/23843

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-80 and 159-174, drawn to methods for increasing nucleic acid synthesis in a cell.
Group II, claim(s) 81-158, drawn to methods for observing increased nucleic acid synthesis.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
Group I is drawn to methods for increasing synthesis of nucleic acids and treating various diseases. Group II is drawn to a research method for studying the response of cells to ultrasound. The group II methods employ different procedures and reagents to achieve an outcome different from that of group I. Thus the two groups do not share a special technical feature within the meaning of Rule 13.2.